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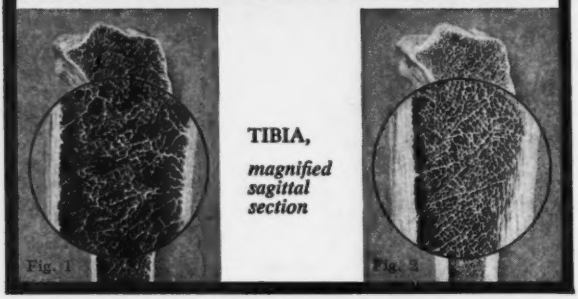
AGING CHANGES THE BONE PICTURE



Estrogen and androgen are vitally concerned with the preparation and recalcification of bone matrix, and this readily explains why declining sex hormone production associated with aging so frequently leads to postmenopausal and senile osteoporosis. Note typical atrophic changes characteristic of postmenopausal osteoporosis (fig. 1), in contrast to normal bone matrix (fig. 2).

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*Reifenstein, E. C., Jr., in Harrison, T. R.: Principles of Internal Medicine, Philadelphia, The Blakiston Company, 1950, p. 655.

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SELECTION OF PHOSPHORUS IN ALLOXAN DIABETES

ILMARI VARTIAINEN* AND MATTI PAASONEN

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OUR earlier experiments demonstrated that alloxan diabetic rats very readily satisfy their need for additional protein with two foods containing phosphoproteins—casein and egg yolk powder (Vartiainen and Paasonen, 1954). We therefore considered it necessary to carry out quantitative experiments on the total phosphorus intake of control rats and of alloxan diabetic rats on self-selected diets.

METHODS

The rats were kept in individual cages and were given a choice of the following foods:
Casein, containing 0.58% phosphorus†
Meat powder, containing 0.43% phosphorus
Egg yolk powder, containing 0.54% phosphorus
Egg yolk powder, fatless, containing 0.85% phosphorus
Egg yolk oil, containing 0.33% phosphorus
Fish powder, containing 0.54% phosphorus
Milk powder, containing 0.58% phosphorus
Semolina, containing 0.20% phosphorus
Dibasic sodium phosphate, in 2% solution. The salt used in the experiments in tables 5 and 6 was offered as an 0.1 molar solution, and sodium pyrophosphate in a 0.05 molar solution was given at the same time.
The animals also received sucrose in a 50% solution and olive oil.

The foods were offered to the rats in various combinations, as shown in the tables. The additional salts, trace elements and vitamins in the diet and other details of the experiments are described in our previous report (Vartiainen and Paasonen, 1954).

RESULTS AND DISCUSSION

The results of our experiments are listed in Tables 1–6. To avoid the use of very low figures the intake of phosphorus by the rats is stated for the entire test period of 28 days instead of daily.

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* Professor Vartiainen was the recipient of a grant from the Sigrid Juselius Stiftelsen and takes this opportunity to express his grateful thanks to this foundation.

† The phosphorus content of the foods was determined by the method of Berenblum and Chain (1938).

TABLE 1. AMOUNTS OF PHOSPHORUS EATEN DURING 28 DAYS BY 12 NORMAL CONTROL RATS AND 10 ALLOXAN DIABETIC RATS (GRAMS OF PHOSPHORUS PER KILOGRAM OF BODY WEIGHT). SIX PROTEIN FOODS AND DIBASIC SODIUM PHOSPHATE OFFERED

	Control rats	Diabetic rats
Phosphorus eaten in:		
Casein	0.8	3.2
Egg yolk powder	0.4	3.5
Meat powder	0.2	1.0
Fish powder	0.1	0.6
Milk powder	0.3	0.5
Semolina	1.0	0.4
Dibasic sodium phosphate	1.0	4.0
Total	3.8	13.2

TABLE 2. AMOUNTS OF PHOSPHORUS EATEN DURING 28 DAYS BY 12 NORMAL CONTROL RATS AND 10 ALLOXAN DIABETIC RATS (GRAMS OF PHOSPHORUS PER KILOGRAM OF BODY WEIGHT). FIVE PROTEIN FOODS AND DIBASIC SODIUM PHOSPHATE OFFERED

	Control rats	Diabetic rats
Phosphorus eaten in:		
Casein or	0.1	1.8
Egg yolk powder	0.1	1.4
Meat powder	0.3	2.2
Fish powder	0.1	0.8
Milk powder	0.6	1.3
Semolina	1.0	1.2
Dibasic sodium phosphate	0.6	2.8
Total	2.8	11.5

TABLE 3. AMOUNTS OF PHOSPHORUS EATEN DURING 28 DAYS BY 6 NORMAL CONTROL RATS AND 11 ALLOXAN DIABETIC RATS (GRAMS OF PHOSPHORUS PER KILOGRAM OF BODY WEIGHT). THREE PROTEIN FOODS AND DIBASIC SODIUM PHOSPHATE OFFERED

	Control rats	Diabetic rats
Phosphorus eaten in:		
Casein	1.6	5.6
Egg yolk powder	0.7	1.3
Meat powder	0.7	1.8
Dibasic sodium phosphate	0.8	1.5
Total	3.8	10.2

TABLE 4. AMOUNTS OF PHOSPHORUS EATEN DURING 28 DAYS BY 6 NORMAL CONTROL RATS AND 4 ALLOXAN DIABETIC RATS (GRAMS OF PHOSPHORUS PER KILOGRAM OF BODY WEIGHT). TWO PROTEIN FOODS AND DIBASIC SODIUM PHOSPHATE OFFERED

	Control rats	Diabetic rats
Phosphorus eaten in:		
Egg yolk powder	0.6	2.4
Meat powder	0.7	3.7
Dibasic sodium phosphate	0.7	0.9
Total	2.0	7.0

TABLE 5. AMOUNTS OF PHOSPHORUS EATEN DURING 28 DAYS BY 9 NORMAL CONTROL RATS AND 9 ALLOXAN DIABETIC RATS (GRAMS OF PHOSPHORUS PER KILOGRAM OF BODY WEIGHT). TWO PROTEIN FOODS, DIBASIC SODIUM PHOSPHATE AND SODIUM PYROPHOSPHATE OFFERED

	Control rats	Diabetic rats
Phosphorus eaten in:		
Casein	1.1	5.8
Egg yolk powder, fatless	0.5	1.6
Egg yolk oil	0.1	0.4
Dibasic sodium phosphate	0.2	0.8
Sodium pyrophosphate	0.4	0.8
Total	2.3	9.4

TABLE 6. AMOUNTS OF PHOSPHORUS EATEN DURING 28 DAYS BY 9 NORMAL CONTROL RATS AND 9 ALLOXAN DIABETIC RATS (GRAMS OF PHOSPHORUS PER KILOGRAM OF BODY WEIGHT). ONE PROTEIN FOOD, DIBASIC SODIUM PHOSPHATE AND SODIUM PYROPHOSPHATE OFFERED

	Control rats	Diabetic rats
Phosphorus eaten in:		
Egg yolk powder, fatless	1.2	6.8
Egg yolk oil	0.1	0.4
Dibasic sodium phosphate	0.1	0.7
Sodium pyrophosphate	0.4	0.9
Total	1.8	8.8

It will be noted that the amounts of phosphorus eaten by the controls were fairly equal. In all the experiments the alloxan diabetic rats ate considerably more phosphorus. Their intake was also relatively equal and about 3–5 times as large as the amounts consumed by the controls. In addition to the phosphorus in the proteins these rats also ate more phosphoric acid salts than the controls, which seems to point to an increased phosphorus requirement in alloxan diabetic rats. No definite differences were seen in the intakes of orthophosphate and pyrophosphate.

SUMMARY

Rats were offered a choice of various combinations of protein foods of known phosphorus content, and phosphoric acid salts.

The intake of phorphorus by alloxan diabetic rats was 3–5 times as great as that of control rats. In addition to a large protein intake they also used more phosphoric acid salts than the healthy rats, which is evidence speaking for an increased phosphorus requirement in diabetics.

No definite differences were seen in the intakes of orthophosphate and pyrophosphate.

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SUCCESSFUL PREGNANCY IN ADRENALECTOMIZED RATS GIVEN SMALL DOSES OF 9α-HALOHYDROCORTISONES[1]

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PREGNANCY in mammals is a biological function which requires a great degree of endocrinological harmony and equilibrium in the body. If rats submitted to total adrenalectomy are kept alike by giving them physiological saline for drinking or by administration of desoxycorticosterone acetate (DCA), they develop changes in the ovaries, an extensive study of which has been recently made by Mandl (1954). On the other hand, the strong activity of the 9α-halohydrocortisones in promoting growth and survival (Borman *et al.*, 1954; Llaurado, 1955) made it desirable to assess the effectiveness of these synthetic steroids in allowing pregnancy when injected to adrenalectomized rats.

METHODS

The animals used in this experiment were the F_1 progeny of a cross between Wistar rats and a strain of black and white hooded rats. They were adrenalectomized when they reached an average weight of 80 gm. and were evenly distributed in groups of eight. All animals were given physiological saline to drink after operation. Twenty four hours later saline was replaced by tap water for drinking and daily administration of 9α-chlorohydrocortisone acetate (Cl-F) and 9α-fluorohydrocortisone acetate (F-F) was commenced. The rats were fed *ad lib.* a pellet diet. Its composition was as follows: bran 10%, pollard 20%, wholemeal flour 35%, skim milk powder 7.5%, meat meal 14.5%, maize meal 10%, bone flour 2.5%, cod liver oil 0.5%. All injections were given subcutaneously using 20% of ethanol in water as solvent. A group of 8 rats was used as control and received only injections of the solvent. The mean survival in this group was 8.6 days and no rat survived longer than 12 days. Previous experiments had shown (Llaurado, 1955) that 10 μg. of Cl-F and 50 μg. of F-F were adequate to keep adrenalectomized rats alive and to promote a satisfactory growth rate. Consequently these doses were adopted in the pregnancy experiment, which was conducted as follows: three females were chosen in each group and mated with males of the same group. Each pair was placed in a separate cage. Parents and offspring were weighed every second day, but only a brief summary of the data are inserted here (Table 1). After parturition the mothers' diet was supplemented with "wet mash"[2] containing a greater proportion of animal proteins, as is usual in the Department.

Received January 17, 1955.

[1] The steroids were supplied by Dr. A. F. Langlykke, Director of Research of The Squibb Institute for Medical Research, New Brunswick, N.J.

[2] Centesimal composition of "mash" supplement: bran 9.52, pollard 19.32, wholemeal flour 24.40, skim milk powder 14.44, meat meal 19.52, maize meal 9.76, bone flour 2.44, cod liver oil 0.60.

TABLE 1. INCIDENCE OF PREGNANCY IN ADRENALECTOMIZED RATS

Pairs		Days of experiment										
		28	39	44	45	49	53	56	58	59	62	72
A Cl-F 10 μg.	Wt. of ♂ (g.)	191	200	206	207	211	212	211	210	200	209	242
	Wt. of ♀ (g.)	161	166	176	182	193	198	202	194	195	190	185
	No.*	—	4	4	4	4	4	4	4	4	4	4
	m. wt. y.	—	4.5	6.8	8.3	12.5	16.5	19.0	20.0	22.0	28.8	55.0
B Cl-F 10 μg.	Wt. of ♂ (g.)	178	213	224	228	231	238	243	247	246	258	275
	Wt. of ♀ (g.)	126	146	133	136	156	161	170	162	158	170	176
	No.	—	—	9	7	6	6	6	6	6	6	6
	m. wt. y.	—	—	3.8	4.2	6.0	9.3	11.5	13.0	14.0	15.0	32.3
C Cl-F 10 μg.	Wt. of ♂ (g.)	221	258	265	269	275	286	285	289	289	294	307
	Wt. of ♀ (g.)	195	212	221	226	248	263	300	308	250	265	276
	No.	—	—	—	—	—	—	—	—	9	9	9
	m. wt. y.	—	—	—	—	—	—	—	—	5.5	7.6	21.0
M F-F 50 μg.†	Wt. of ♂ (g.)	147	169	167	167	172	157	DEAD	—	—	—	—
	Wt. of ♀ (g.)	121	147	160†	160	175	165	186	184	185	195	213
	No.	—	—	—	—	—	5	5	5	5	5	5
	m. wt. y.	—	—	—	—	—	4.2	5.4	6.0	8.0	9.2	18.0
N F-F 50 μg.†	Wt. of ♂ (g.)	204	235	249	250	255	262	273	274	268	282	307
	Wt. of ♀ (g.)	148	176	165†	182	166	165	163	159	153	DEAD	—
	No.	—	—	3	DEAD	—	—	—	—	—	—	—
	m. wt. y.	—	—	3.6	—	—	—	—	—	—	—	—
O F-F 50 μg.†	Wt. of ♂ (g.)	163	180	189	186	184	172	165	DEAD	—	—	—
	Wt. of ♀ (g.)	147	175	188†	195	179	191	203	199	184	185	180
	No.	—	—	—	—	7	7	7	DEAD	—	—	—
	m. wt. y.	—	—	—	—	4.2	4.9	6.1	—	—	—	—

* No. = Number in litter. The first entry is on the day of birth.
m. wt. y. = Mean weight of young in grams.
† After 44 days owing to shortage of material DCA (50 μg.) had to be substituted for the F-F.

RESULTS AND CONCLUSIONS

Mothers treated with Cl-F produced live healthy litters (Table 1) whose average weight, date of eruption of the teeth and opening of the eyes were within the range described by Farris and Griffith (1949). Three members of one litter (pair B, Table 1) died soon after birth, but no significance is attached to it, because the loss of some in the first litter of a rat is a common occurrence. Furthermore, the handling of litters for counting and weighing, and the circumstance that some rats were rather young when they become pregnant, may also probably account for some of these losses. It can, therefore, be concluded that adrenalectomized male and female rats given Cl-F (10 μg./day) are capable of producing healthy litters and that the subsequent development of these litters follows the normal pattern.

Compound F-F allowed fecundation and pregnancy, but the supply was exhausted before the end of pregnancy and had to be replaced by 50 μg. of DCA (Table 1). All litters were born alive, but two of them subsequently died. The remaining litter survived and developed normally.

The fact that successful pregnancy was achieved and normal litters were born from adrenalectomized rats is further evidence that Cl-F and F-F are highly effective in restoring the loss of homeostasis of the body

after adrenalectomy. It shows too that these compounds in the dosage used do not interfere with pregnancy.

SUMMARY

Adrenalectomized male and female rats treated with 9α-chlorohydrocortisone in the dosage of 10 μg./day remain alive and apparently healthy for an indefinite period and are able to produce and rear normal litters. Treatment with 9α-fluorohydrocortisone (50 μg./day) is probably equally effective, but owing to insufficient material treatment had to be stopped before term and some litters died soon after birth.

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ENDOCRINE RELATIONSHIPS IN MALES OF A RELATIVELY INFERTILE STRAIN OF WHITE LEGHORN FOWLS

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OVER a period of 15 years, one strain of White Leghorns (C) at this laboratory has been consistently less fertile than two other strains (K and S) although all three have been maintained under identical conditions. The birds of the three strains are intermingled in the incubators and brooders, on the rearing range, and in the laying houses. During the breeding season, representatives of the infertile strain are given the same kinds of breeding pens as the others, and in the same buildings. Since the environment is uniform for all three strains, it is clear that the consistently low fertility of Strain C is genetically determined.

From 1943 to 1953 the average fertility of all eggs incubated from cockerel-test matings (*i.e.*, no prior information regarding the fertility of the birds used) in Strains C, K, and S was 64, 91, and 86%, respectively. Thus, if the fertility in Strains K and S be accepted as normal for single-male matings in the species, then the genetic abnormality of Strain C reduces its fertility by 25 to 30%.

For convenience in tables and in text, Strain C will be designated as the "infertile" strain. Strictly speaking, this term is a misnomer, for, although this strain is subnormal in fertility, it is far from being completely infertile.

In earlier studies of this condition (Gowe and Hutt, 1949) the females were incriminated as being chiefly responsible, but later work has shown that the males of Strain C are also abnormal. This paper reports evidence that in these males of the infertile strain the functioning of certain endocrine glands differs from that in males of the fertile strain (K).

WEIGHTS OF VARIOUS ORGANS

Testes. Having noticed in 1949 that cockerels of the infertile strain showed conspicuous development of the comb at an early age, combs

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and testes were weighed from samples of the C and K lines at 63 days (51 males) and at 86 days (77 males). At both ages testes in the infertile strain were about three times as large, and combs twice as large, as in the fertile strain, all differences being statistically significant ($P = <.001$). At 63 days, the proportion of males showing combs lopped over to one side (because of their large size) was 72% in the infertile strain, but only 8% in the fertile one.

To determine the time and rate at which precocious gonadic develop-

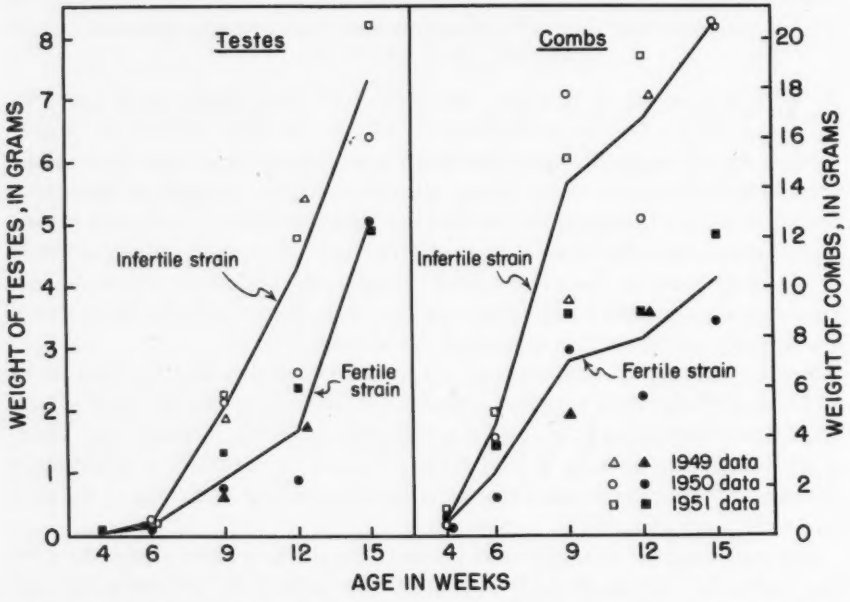

FIG. 1. Weights of the testes and of the combs from males of Strains C and K at five different ages. Each point on the graphs represents the average for different groups of males, ranging in number from 11 to 39 males per group. The precocious development of the testes and the combs in the infertile strain (C) is clearly evident.

ment proceeded in the infertile strain, random samples of males from Strains C and K were taken in 1950, and again in 1951, at 4, 6, 9, 12, and 15 weeks. In 1950, the number of males used at each age was 20 per strain, except at 4 weeks when, because of their small combs, only 17 males of Strain K could be recognized. In 1951, the number per strain varied from 11 to 19 at different ages.

Mean weights of testes in all samples of Strains C and K in 1949, 1950, and 1951 are shown in Figure 1. They reveal that even by 6 weeks of age the testes were larger in the C strain than in the fertile one. The difference increased at later ages and even at 15 weeks of age testes of the C males were larger than in the other line. In 1950, these differences were highly

significant (P = <.01) at 6, 9, and 12 weeks. In 1951, with slightly smaller numbers, the differences were significant at 12 and 15 weeks.

Comb. Corresponding with this precocious development of testes in Strain C, combs of the males in that line were bigger even at 4 weeks, than in the other (Fig. 1). All combs were cut off as close to the skull as possible and weighed. Considering all three years' data, from 6 to 15 weeks of age the combs from males of the infertile strain in different lots were from 1.7 to 2.5 times as large as combs from the fertile strain. All these differences were statistically significant, 9 out of 10 being so at the level: P = <.01.

As Figure 1 shows, the combs grew more rapidly in both strains at 4 to 9 weeks. After that age, further growth proceeded at a much slower rate in both strains, but the remarkable difference established by 9 weeks was maintained. All of these cockerels were weighed just prior to slaughter, with no significant differences being found between the two strains at any of the ages studied.

Pituitary.

From cockerels used in 1950 and 1951 the anterior lobes of the pituitaries were obtained and weighed in the following manner. The head of the bird was removed in such a way that the atlas separated from the skull, leaving the *foramen magnum* open. After cutting off the remaining skin on the top of the head, the tip of a pair of bone-cutting forceps was inserted into the *foramen magnum* and the top of the skull cut away. The soft brain tissue was then shoved forward to expose the floor of the cranium and, with the bone forceps, the floor of the cranium was cut along the midline as far forward as the prominence immediately posterior to the *sella turcica.*

The two halves were then separated and the pituitary brought into full view. The entire organ, with a small portion of the hypothalamus, was removed and dropped into Dawson's fluid (9 parts saturated aqueous solution of mercuric chloride to 1 part 10% formalin). After 24 hours it was transferred to 70% alcohol. Then, after cutting away the *dura mater,* the anterior lobe was peeled from the remaining tissue. No cutting was necessary to separate the anterior lobe, and thus the accuracy of the technique was enhanced. The anterior lobe was weighed in a weighing jar on a chainomatic balance. Physiological saline, because of its low volatility, was used to reduce evaporation in the weighing jar.

At all 5 ages, in both years, the anterior lobe of the pituitary was significantly heavier in males of the infertile strain (Table 1). The series of pituitaries obtained in 1950 were grouped by strain and age, but were not identified as to individual donor. Thus their individual weights could not be related to the individual body and organ weights of the cockerels from which they had been obtained.

Interrelationship of organ weights. To study the relationships among the various organs within each strain and age, Wright's method of path coefficients (1934) was applied to the data for 1951. To save space the details of this procedure are not given here. However, the dependence of testes weight upon pituitary weight at 4 and 6 weeks of age was found to be greater in the infertile strain than in the fertile strain (standard partial

TABLE 1. A COMPARISON OF WEIGHTS OF THE ANTERIOR LOBE OF THE PITUITARY FROM MALES OF STRAINS C AND K

Population	Age, weeks	Infertile strain (C)		Fertile strain (K)		C–K	
		Birds, no.	Mean wt., mg.	Birds, no.	Mean wt., mg.	t value	F value[2]
1950[1]	4	13	2.37 ± .169	12	1.93 ± .073	2.33*	—
	6	18	2.54 ± .086	18	2.02 ± .115	3.63†	—
	9	16	4.48 ± .186	17	3.21 ± .102	6.08†	—
	12	19	5.66 ± .225	17	3.95 ± .189	5.74†	—
	15	19	6.23 ± .378	15	4.71 ± .206	3.28†	—
1951	4	19	2.69 ± .063	19	2.27 ± .072	—	18.88†
	6	15	3.79 ± .166	15	3.11 ± .101	—	8.33†
	9	15	5.26 ± .249	15	4.43 ± .228	—	10.51†
	12	11	6.08 ± .385	11	4.34 ± .140	—	20.02†
	15	13	7.15 ± .366	13	5.51 ± .355	—	16.07†

[1] These weights have been corrected for strain differences in body weight.
[2] Analysis of covariance; the variance due to linear association with body weight has been removed.
* P = <.05.
† P = <.01.

regression coefficients of +.38 and +.26 vs +.05 and −.02). This suggests that in Strain C a physiological relationship between the pituitary and testes has been established even at these early ages, and that the large pituitaries of the young males are actually releasing gonadotrophic hormones.

STRUCTURE AND FUNCTION OF ENDOCRINE GLANDS

Histology of testes. In 1950 cockerels showing combs of average size for their strain and age were selected for histological study of their testes. Tissues fixed in Bouin's fluid were sectioned at 8μ, and stained with either haematoxylin and eosin or Mallory's triple connective-tissue stain.

At 4 weeks of age no difference was evident between the strains in development of testes. Thereafter the testes in Strain C showed more advanced stages of spermatogenesis than those of Strain K. The differences between strains were determined quantitatively by measuring diameters of 40 representative seminiferous tubules from each strain at each age. For this purpose, 5 fields were selected from sections of each of 8 testes per strain. One field was chosen from each quadrant of the section, and another near the center. In each of these fields, one tubule was selected at random for measurement, using a pointer in the eye-piece of the microscope to make the selection. An ocular micrometer was used for measuring the diameters of the tubules and the data were converted to microns.

Corresponding to the greater size of testes in Strain C, the seminiferous tubules were much larger in that strain than in Strain K at all ages after 4 weeks. At 6, 9, 12, and 15 weeks the mean diameters of 40 tubules were 73, 180, 193, and 235μ in the infertile strain, compared with 52, 101, 83, and 162 in the normal one. By 9 weeks of age, free spermatozoa in the lu-

mina of tubules were evident in 2 of the 8 testes examined from Strain C, but that stage was not found in the fertile strain until 15 weeks.

In general the development of spermatogenic activity was closely correlated with the size of the gland. Testes appeared to be developing normally in both strains but were doing so precociously in the infertile strain, C.

Endocrine activity of testes. As is well known from the work of many previous investigators, development of the comb in young male chicks is an indication that the testis is producing testosterone. Moreover the size of the comb in growing birds is a measure of the amount of male hormone produced. In these studies, the correlation between size of comb and size of testes was measured in 1950 in each strain at 4, 6, 9, 12, and 15 weeks. All 10 correlations were positive, ranging from +0.38 to +0.79, the average being +0.59.

It is recognized that, whereas the histological studies just reported have revealed that precocious spermatogenesis is characteristic of Strain C, the accompanying enlargement of the seminiferous tubules cannot be taken *per se* as a measure of the production and secretion of testosterone. The original extensive evidence of Benoit (1922, 1924, 1929) that male hormone is produced by the interstitial cells in the intertubular tissue has been confirmed by Sluiter and van Oordt (1946, 1947). However, all these investigators found that, contrary to appearances, when the testes and comb increase during puberty, the volume of interstitial cells also increases. One may therefore accept comb size as a measure of the output of testosterone. The fact that most males were recognizable at 4 weeks in Strain C, but relatively few in Strain K, shows that testes in the former were secreting more hormone at that early age before spermatogenic activity was evident histologically in either. Altogether the data show that the infertile Strain C is characterized by a remarkable precocious activation of the male gonad.

Other observations. To determine whether the gonadotrophic potency per unit of pituitary tissue differed in the two strains, measured amounts of dried tissue from 15- and 16-week-old donors were assayed. Three consecutive, daily, subcutaneous injections (each consisting of 5 mg. of pituitary powder in 0.3 ml. saline) were made into inbred females, 23–25 days old, of the Webster strain of Swiss mice. Twenty-four hours after the last injection the mice were sacrificed, and the uteri removed and weighed. Uteri of the injected mice were approximately twice as large as the controls (injected with saline), but no quantitative differences were observed between pituitary material from the C strain and that from the K strain. However, since the yield of dried powder was, on the average, more than a third greater from each male of Strain C (1.83 vs. 1.34 mg.) it seems probable that the total amount of gonadotrophic hormone secreted per bird was correspondingly greater in the males of the infertile strain (C).

Tissue sections of the pituitary, thyroid, and adrenal from males of each

strain at each age studied revealed no differences in their histological appearance.

STRAIN DIFFERENCES IN AGE AT FIRST EGG

If early sexual development in males of the infertile strain is due to a precocious production of gonadotrophic hormones, the females of that strain might respond similarly by commencing egg production at an earlier age than do females from the fertile strain. Examination of the records shows that the females of the infertile strain (C) began laying about 6.6 days earlier than did those from the fertile one (K) (Table 2). The differ-

TABLE 2. STRAIN DIFFERENCES IN MEAN AGE AT FIRST EGG

Year	Infertile strain (C)			Fertile strain (K)			Difference K–C days
	Sires, no.	Daughters, no.	Mean age at 1st egg, days	Sires, no.	Daughters, no.	Mean age at 1st egg, days	
1946	18	726	186.4	20	962	193.5	+ 7.1
1947	13	746	184.8	14	906	196.4	+11.6
1948	9	518	181.1	9	783	185.6	+ 4.5
1949	17	821	176.9	16	1210	181.7	+ 4.8
1950	18	695	183.1	17	964	188.5	+ 5.4
1951*	4	379	160.5	6	662	173.2	+12.7
1952	13	755	163.1	16	1075	170.9	+ 7.8
1953	11	761	165.5	22	1308	172.6	+ 7.1
	103	5401	175.8	120	7870	182.4	+ 6.6

* Data limited to progeny of previously tested sires.

ence was quite consistent from year to year. In both strains the age at first egg was 20.9 days less in 1953 than in 1946. This trend towards a younger age at first egg is primarily a consequence of selection.

That strains of fowls differ in age at first egg is well known. To the best of our knowledge no association of such variations with differences in fertility has previously been noted, and the significance in the present case has yet to be determined.

DISCUSSION

The authors are aware of the fact that the size of an endocrine organ may be unrelated to its physiological activity. However, the significantly larger anterior lobe of the pituitary in the male of the infertile strain is associated with significantly larger and more precociously developing testes and also with an abnormally large comb which begins development very early in the life of the young cockerel. These relationships are in accord with accepted interpretations of the hormonal control of the gonad and the secondary sexual characters. Although there were no apparent strain differences in the microscopic appearance of the anterior pituitary, it is possible that differences in physiological activity could exist without ap-

parent changes in the histological appearance. The attempt to assay the pituitary for gonadotrophic hormones did not reveal any great differences per unit of gland. However, it was shown that anterior lobes from males of Strain C were approximately one-third larger. This does not necessarily mean that the organ secretes for the use of the body that much more gonadotrophic hormone, although the greater response of the testes would suggest that this is the case.

The abnormal development of the comb in males of Strain C might be brought about by a greater production of luteinizing hormone (LH) by the anterior pituitary, which, in turn, stimulates the growth of the Leydig cells and the production of androgen (Nalbandov, *et al.*, 1946). The most obvious difference in the histology of the gonads from very young males of the infertile strain, compared to those of the fertile strain, was shown to be a marked enlargement of the seminiferous tubules and an earlier and more extensive spermatogenesis. This in itself would indicate the action of FSH rather than LH.

Baird, *et al.* (1952), reported on a somewhat similar problem dealing with the relationship of the growth hormone to marked differences in the rate of growth in two lines of swine. In this case the pituitaries did not differ in weight, but they did differ in the amounts of growth hormone per unit of tissue.

The adrenal cortex is known to produce androgenic substances which stimulate secondary sexual characters, in this case the comb development. That abnormal functioning of the adrenal is not a primary factor in our infertile strain would seem evident for two reasons. The relative proportions of medullary and cortical tissues were similar for both strains at all ages studied and no obvious histological differences were recognized. Abnormal production of androgen by the adrenal cortex is usually followed by a reduction in gonadotrophic hormone production by the pituitary and in turn a reduction in the size of the gonads. Since this was not the case, it suggests that the androgen responsible for the excessive comb development was of testicular origin.

The possibility remains to be investigated that the infertile strain may differ from the fertile one in the response of gonad and comb to hormones rather than in the amounts available. Callow and Parkes (1935) and Dorfman (1948) have shown that the comb of the Leghorn cockerel is several times as sensitive to androgen as that of the Rhode Island Red or the Barred Plymouth Rock. More recently Campos and Shaffner (1952) showed sire- and dam-family differences in response of New Hampshire chicks to testosterone propionate and to stilbestrol.

SUMMARY

Among young males from two strains of White Leghorns differing in fertility, it was found that, while the growth of the body was similar, those

from the relatively infertile strain had disproportionately larger pituitaries, testes, and combs. Spermatogenesis was closely correlated with size of testes. On the basis of organ weight, the hormonal stimulation of the gonad appeared to be established very early in the infertile strain.

No strain differences were observed in the microscopic structure of the pituitary, adrenal, or thyroid.

The amount of gonadotrophic hormone per unit of pituitary tissue (as determined by its effect upon the uteri of young mice) was equal in the two strains at 15 to 16 weeks of age, but the total weight of the anterior pituitary was about one-third larger in the infertile strain.

Sexual maturity, measured by age at first egg, was consistently earlier for the females of the infertile strain, although this could be a phenomenon unrelated to the precocious sexual development of the males.

These observations suggest that the larger pituitary of the young males of the infertile strain may provide an excess of gonadotrophic hormones which induce a precocious development of the testes, and thus an early development of the comb. Whether or not such changes in these young males are responsible for the relative infertility of their strain remains to be determined.

Acknowledgment

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THE GROWTH HORMONE CONTENT OF HUMAN PLASMA[1]

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THE present investigation was undertaken in an effort to assay the growth hormone content of body fluids. Reports to date show a successful assay in only one acromegalic patient (Kinsell, 1950). The thickness of the tibial epiphyseal cartilage in the immature hypophysectomized rat is the most sensitive of the currently available methods and, therefore, this method was employed.

It was not considered worthwhile to attempt to set up a model for fractionation of plasma based on ability to recover hypophyseal growth hormone added to plasma before fractionation because of the different solubility characteristics of growth hormone prepared by various methods from pituitaries of different species. Instead it was thought advisable to attempt first the measurement of the growth hormone content of whole plasma. After preliminary trials the following standardized procedure was employed. Immature rats of the Sprague-Dawley strain (75 to 85 gm. in weight) were hypophysectomized, permitted to rest for two weeks, and then for four days given injections of a solution of either growth hormone or plasma. The preparation of growth hormone used was of bovine origin, standardized by us in terms of the Armour Standard 22KR2. The blood was collected in heparinized syringes and spun down immediately in a refrigerated centrifuge. Plasma from this was separated into aliquots for each day's injections and frozen in the deep freeze so that the supply for the day was contained in a single small tube which could be thawed immediately before the first injection. Each rat received 0.2 ml. of this plasma twice daily for 4 days. In order to test for recovery of added growth hormone activity, various amounts of the hormone were added to the plasma being assayed, usually in two different amounts.

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The standard procedure of Greenspan (Kinsell, Michaels, Li, and Larsen 1948) was employed throughout the present studies. Autopsies were performed on the fifth day, the tibias were removed, fixed in formalin, washed in running water for 30 minutes, soaked in acetone for one hour, and washed again for 30 minutes. The tibias were then split sagittally at the proximal end and placed in a fresh 2% solution of silver nitrate for 1 to 2 minutes. After this, they were placed in distilled water and exposed to strong light until a black line appeared at the edge of the epiphyseal cartilage. They were then placed in a 10% solution of sodium thiosulfate for at least 30 seconds, washed for one half hour, placed in 80% alcohol and kept in this solution in the dark.

The thickness of the epiphyseal cartilage was measured by ocular micrometer under a binocular dissecting microscope. Ten readings to the nearest graduation were taken at regular intervals across the cartilage plate and averaged to obtain one mean reading in microns. The mean cartilage widths of all the tibias from rats injected with a given plasma preparation were averaged to ascertain the mean for that particular preparation. To complete the assay, the concentration of growth hormone was calculated and expressed in gammas of the Armour Standard 22KR2.

When growth hormone is added to plasma and administered to immature hypophysectomized rats, there is an increase in tibial cartilage thickness. When the values for tibial cartilage thickness are plotted on rectangular coordinates a line is obtained which is parallel to the line obtained when the growth hormone is administered either in saline or in bovine albumin. Such a line must be plotted on rectangular coordinates since some growth hormone is contained in the plasma. If a semi-logarithmic plot were used and the lines remained parallel then increasing values must be assigned to the growth hormone in plasma at the higher added amounts of growth hormone.

Since the major solutes of plasma are proteins, studies were conducted to determine whether protein alone could account for the increase in cartilage thickness. Crystalline bovine serum albumin was employed for this purpose. A concentration of 7 gm. per 100 ml. was used to approximate the protein concentration of plasma. Solutions obtained by adding concentrations of 12.5 γ to 50 γ of growth hormone to this albumin solution were assayed and the results were plotted. Using standard statistical methods, this curve did not differ significantly from the corresponding curve obtained with a saline solution of growth hormone. Therefore, it was concluded that the increase in cartilage thickness following injection of human plasma could not be attributed to the protein content of the plasma. The constituent of plasma, other than growth hormone, which might account for the results obtained is thyroxin. It has been previously shown by others (Li, 1953) and confirmed by us that if animals are given thyroxin for two weeks immediately after hypophysectomy it is possible to produce

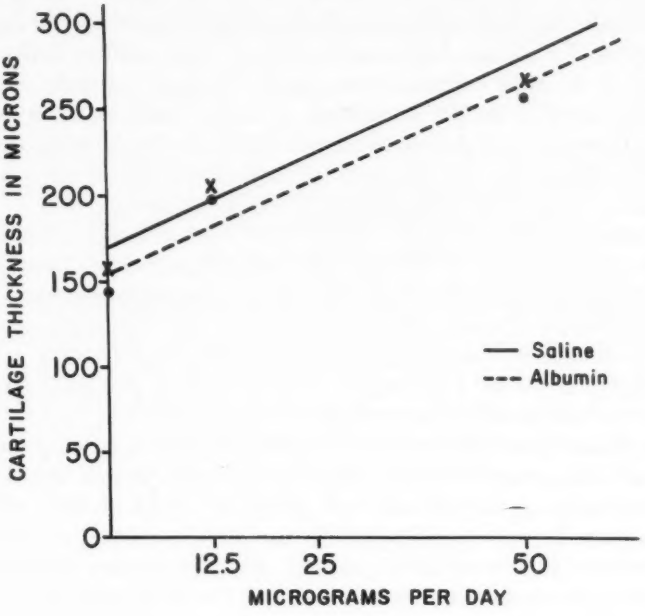
CARTILAGE THICKNESS IN MICRONS
300
250
200
150
100
50
0
12.5
25
50
MICROGRAMS PER DAY
Saline
Albumin

FIG. 1

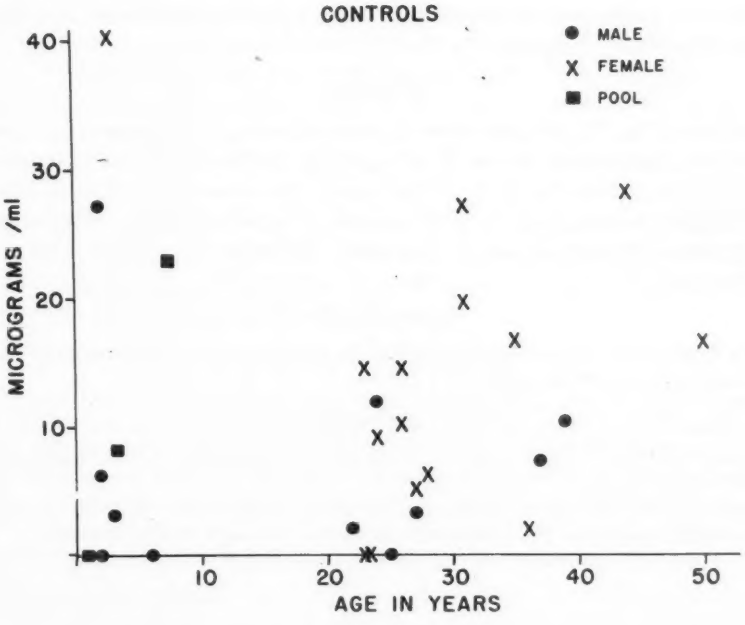
CONTROLS
MALE
FEMALE
POOL
MICROGRAMS /ml
40
30
20
10
10
20
30
40
50
AGE IN YEARS

FIG. 2

an increase in cartilage thickness. However, we have observed no increase in thickness of cartilage following treatment with sodium L-thyroxin for four days in dosages varying from minute to toxic amounts. Since these two major possibilities for increasing cartilage thickness in hypophysectomized immature rats do not seem to interfere in the four-day assay, it is our belief, at least for the present, that the results being reported are indeed due to the growth hormone content of the plasma.

The standard curve (Figure 1) used as a reference was calculated from cartilage measurements in 438 rats. Statistical analysis of these data indicates that the curves representing the action of growth hormone dissolved in either saline or albumin solution are statistically straight lines and that they are, moreover, parallel and do not differ significantly from each other. Figure 2 shows the results obtained when plasma from normal individuals is assayed by this method.

Plasma from three individuals, considered to have active acromegaly on clinical and laboratory evidence, showed elevated plasma titers of tibial line increasing material (41, 61, and 55 μg./ml., respectively). Following deep roentgen therapy to the pituitary the plasma content of growth hormone decreased in two of these patients. Plasma was assayed from seven "burned out" acromegalic patients who had shown no evidence of growth for several years. The values obtained varied from 0 to 16 μg./ml. No tibial line increasing material was demonstrated in the plasma from six individuals with panhypopituitarism which was judged complete on clinical and other laboratory grounds.

SUMMARY

An assay for the growth hormone content of human plasma is proposed, based on the increase in the tibial cartilage of the immature hypophysectomized rat. Values for normal individuals in terms of the Armour Standard 22KR2 varied from 0 to 40 μg./ml. No such material was found in panhypopituitarism whereas excessive amounts were found in active acromegaly.

Acknowledgment

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INFLUENCE OF CITRIC ACID CYCLE INTERMEDIATES ON THE *IN VITRO* METABOLISM OF CORTICOSTEROIDS IN ASCORBIC ACID DEFICIENCY[1]

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NUMEROUS studies designed to determine the role of ascorbic acid in adrenal cortex function have employed parameters such as urinary corticosteroids, urinary 17-ketosteroids, liver glycogen, and blood leukocytes as indices of adrenal activity. In many cases these observations were interpreted without regard to possible alterations in corticosteroid metabolism by the tissues of the ascorbic acid deficient animal, or in changes in the target organs in this condition. In recent work we pointed out that ascorbic acid nutrition influences the metabolism of the side-chain, and the Δ^4,3-ketone structure of the nucleus, of cortisone and desoxycorticosterone. It was shown that *in vitro* preparations of liver slices of ascorbic acid-deficient animals metabolize greater amounts of the 17-hydroxyl,-20,21-ketol grouping than do control liver tissues (Bacchus, 1953a). With employment of a superior technique it was also shown that ascorbic acid excess derpresses the metabolism of the side chain, as well as of the $\alpha\beta$ unsaturated structure of the nucleus, by normal liver tissues (Bacchus, 1953b).

This paper presents data on the increased metabolism of the 17-hydroxyl,20,21-ketol side chain of cortisone by liver slices of ascorbic acid-deficient guinea pigs. The studies also demonstrate that certain intermediates in the citric acid cycle alter this process.

MATERIALS AND METHODS

Animals: Liver tissues were obtained from female guinea pigs. These animals were obtained from a commercial dealer (Hilltop Caviary, Scottdale, Pa.) at initial weight about 300 gm. The animals were maintained in our quarters for about one week prior to being submitted to the dietary fares of the experiment. One group (I) of animals was given an *ad libitum* diet of Purina Rabbit Chow (containing at best only traces of ascorbic acid; see Bacchus and Heiffer, 1953), and tap water without any supply of greens or ascorbic acid. This fare has been shown to be rapidly scorbutigenic in the guinea pig.

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The control group (II) consisted of similar animals which were given a diet of Purina Rabbit Chow and tap water, along with liberal amounts of fresh lettuce. This fare has been observed to prevent the development of ascorbic acid deficiency. The intake of Chow by this group of animals was regulated on the basis of the intake of group I so that the total food intake, and the body weights of these groups were comparable throughout the experiment. Early in the experiment observations were also made on normal guinea pigs fed *ad libitum* on a complete diet. These animals suffered no weight losses. After periods on these fares, animals were anesthetized with nembutal, laparotomy was performed, and the liver was exicsed.

Preparation of Liver Slices: The liver was blotted with filter paper. Thin slices were then cut and weighed on a Roller-Smith torsion balance. The incubation preparations contained 60±2 mg. slices. These slices were not washed or otherwise treated since it be-

TABLE 1. SAMPLE SERIES OF INCUBATION PREPARATIONS FOR EXPERIMENT TO DETERMINE THE INFLUENCE OF ASCORBIC ACID-DEFICIENCY ON THE ABILITY OF THE LIVER TO METABOLIZE THE 17-OH,20,21-KETOL STRUCTURE OF CORTISONE

Sixty milligram slices of liver tissue, fresh and unwashed, were taken from paired-animals —one on a scorbutigenic diet, the other pair-fed on a complete diet. One slice from each animal incubated with buffer only; one slice from each animal incubated with 200 μg. cortisone in buffer. Extraction with chloroform. One-fourth aliquots used for Porter-Silber test and sulfuric acid blanks. Optical densities for Porter-Silber chromogens corrected for H_2SO_4 blanks. Optical densities ascribable to steroid determined after correction for liver blanks. In addition to the incubation series above, a preparation of liver+cortisone, extracted without incubation, was also included.

Incubation preparations	Buffer only	Steroid in buffer	Control liver slice with buffer	Control liver slice with steroid	Deficient liver slice with buffer	Deficient liver slice with steroid
Sulfuric acid blank =	0.002	0.001	0.004	0.002	0.002	0.002
P-S chromogen =	0.003	0.375	0.004	0.300	0.003	0.240
Corrected density =	0.001	0.374	0.000	0.297	0.001	0.238
μg. steroid recovered	0	187	0	148	0	119
μg. metabolized	—	(13)	—	52	—	81

¼ aliquots used for analyses; Standard = 25 μg. Cortisone = 0.200.

came apparent that storage, or washing and rinsing, altered the ability of the slices to metabolize the steroid.

Incubation preparations: The sixty mgm. slices were incubated in 3.0 ml. phosphate-saline buffer (pH 7.40, Clark-Lubs) with 200 micrograms of cortisone. The method of preparation of the steroid solution was presented earlier (Bacchus, 1953b). Each experiment involved liver slices from control animals, and from ascorbic acid-deficient animals, incubated with cortisone. Blanks consisted of incubation preparations of liver tissue without cortisone, preparations of liver tissue extracted without incubation, as well as non-incubated liver-cortisone preparations. A complete sample experiment is included in Table 1. These blank preparations enabled us to determine the possible contamination of the analytical procedure by liver tissue, the recovery by the extraction and analytical methods, as well as the specificity of the analytical method. The preparations were incubated at temperature of 37.5° C., for three hours; the tubes were open to atmospheric air.

Co-factors: The influence of some citric acid cycle intermediates was determined by the addition of 0.03 M amounts into individual incubation preparations. Adequate blanks of these substances were negative for Porter-Silber chromogenicity. The intermediates used were pyruvate, citrate, oxalacetate, α-ketoglutarate, succinate, fumarate, and malate. The influence of ascorbic acid added *in vitro* was also studied.

Extraction: At the end of the incubation (3 hr.) the entire preparations were extracted with three volumes of warm chloroform. Ten ml. chloroform was added to each tube, and extraction carried out as described (Bacchus, 1953b).

Analytical Procedure: The Porter-Silber (1950) method was employed for detection of the 17-OH,20,21-ketol side chain. The original method as recommended by Porter & and Silber (1950) was employed, including the use of sulfuric acid blanks to correct for interfering chromogens; these were negligible (Table 1).

RESULT

1. *Optical density readings of liver slice blanks, and recovery of cortisone under conditions of the experiments:* Our previous application of the methods employed here showed that purification beyond washing was not necessary. It seems useful, however, to consider the amount of contamination observed in conduct of the analytical procedure. A sample experiment is presented in Table 1. The series of incubation vessels, and the optical density readings, at 410 mμ, for Porter-Silber chromogens and for sulfuric acid blanks are also presented in the table. It is seen that the optical density readings of extracts ($\frac{1}{4}$ aliquots) of the control vessels, as well as of the chemical blanks, are negligible. Throughout our entire series of experiments no liver tissue blank (liver+buffer) when submitted to the Porter-Silber method gave an optical density reading over 0.004 for the $\frac{1}{4}$ aliquot used. This is true of the sulfuric acid blanks, as well as of the aliquots on which the phenylhydrazine reaction was done. The optical density readings on the blanks throughout the experiment ranged between 0.000 to 0.004. These data further confirm our earlier demonstration that this method of *in vitro* study is accurate and reliable. It is also seen from Table 1 that the recovery of cortisone from a preparation of 200 μg. cortisone in buffer only (i.e., in the absence of liver) reaches 94%. Preparations of cortisone+liver, extracted immediately (no incubation) revealed about 95–102% recovery. These data demonstrate also that the method of analysis is highly specific under these conditions, entailing minimal contamination by liver tissue.

2. *Metabolism of the 17-OH,20,21-ketol structure by normal liver tissue:* Liver slices of normal guinea pigs metabolize this structure to the extent of 52 ± 0.50 (S.E.) micrograms per 60 mg. slice over the three hour period. This value is based on observations from ten normal female guinea pigs. Triplicate incubation preparations from five of these animals revealed a mean of 52 μg. with a Standard Error of 0.49. (Earlier studies revealed that the rat liver metabolizes 100–115 μg of this structure per 60 mg.) It was also found that the ability of the liver slices to metabolize this structure was not appreciably influenced by the substitution of 1.33 mg of sodium ascorbate for the *ad libitum* feedings of fresh greens. This dose of ascorbic acid was found to be sufficient to maintain the control animals (Bacchus, 1953a).

3. *Metabolism of the 17-OH,20,21-ketol structure by liver slices of guinea*

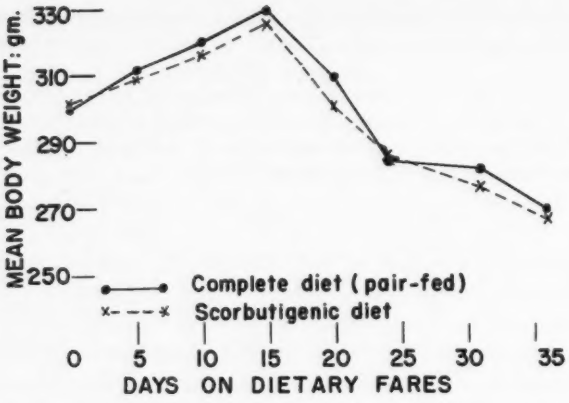

FIG. 1. Body weights of female guinea pigs, on scorbutigenic fare, and on pair-feeding of complete diet.

pigs on a scorbutigenic regime, and on a control (pair-fed) diet: Liver slices were obtained from animals on the dietary fares for 5, 10, 15, 24, 31, and 36 days. The animals on the scorbutigenic fare for 31–36 days were moribund. Earlier studies indicated that the mean survival period on the scorbutigenic fare is 30–31 days. Data on the body weights of the animals are presented in Figure 1. The data on steroid metabolism by the liver slices are presented in Figure 2. It is seen that the liver slices of the control animals metabolize the 17-OH,20,21-ketol structure to the extent of 52 μg.

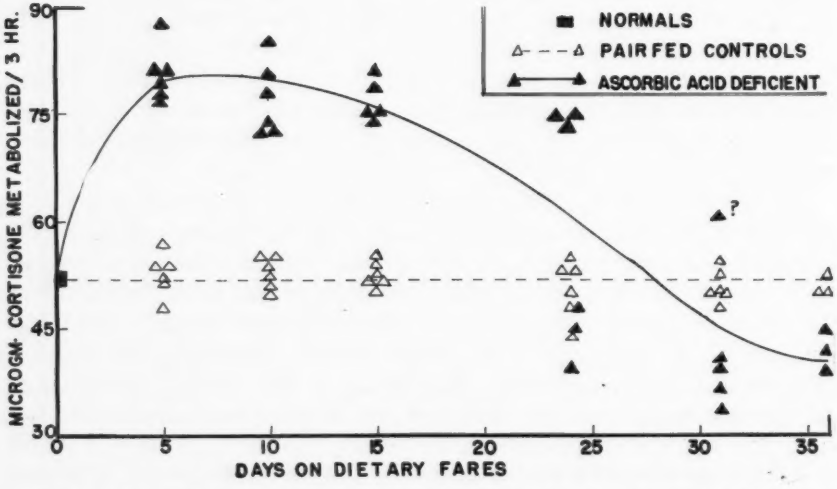

FIG. 2. Metabolism of the 17-OH,20,21-ketol side chain of cortisone by liver slices from ascorbic acid-deficient, and control, guinea pigs. Individual values are presented. Descriptions of preparations in text.

per 60 mg slice. It is apparent that the degree of inanition and weight loss associated with the pair-feeding technique failed to appreciably influence the ability of the liver to metabolize this structure. The extent of metabolism after 5 days on the scorbutigenic fare was 81 μg, after 10 days, 77 μg., after 15 days, 77 μg., after 24 days, 62 μg., after 31 days, 48 μg, and after 36 days, 43 μg. The extent of metabolism on days 5, 10, and 15, were significantly greater than the respective controls ($P < 0.01$). Although the mean metabolism on day 24 was 62 μg., this increase was not statistically significant. These data indicate that the response of the liver to the deficient diet changes with the duration on the fare. The period up to 24 days on the diet was associated with an increased metabolism of the structure. During the period 31–36 days, when the animals were moribund, there was a significant decrease in the rate of metabolism of the 17-OH,20,21-ketol structure.

4. *Influence of treatment of the liver slices prior to slicing on the ability to metabolize the 17-OH,20,21-ketol structure:* Liver slices were obtained from a control guinea pig, and from one animal on the scorbutigenic fare for 15 days. The data are presented in Table 2. The liver slice of the control animal metabolized the structure to the extent of 53 μg. over the 3 hr. period. The tissue of the ascorbic acid-deficient animal metabolized 75 μg. After rinsing the slices in three changes of saline-buffer for 4 to 5 minutes, the control liver slice metabolized 40 μgm. of the structure. Similar treatment resulted in the metabolism of 50 μg. by the liver of the ascorbic acid-deficient animal. Longer storage resulted in further alterations, with equalization, of the abilities of the liver slices to metabolize the 17-OH, 20,21-ketol structure.

TABLE 2. EFFECT OF RINSING AND STORAGE OF LIVER TISSUES ON THE ABILITY TO METABOLIZE THE 17-OH,20,21-KETOL STRUCTURE OF CORTISONE

Treatment of liver	Micrograms loss/60 mg./3 hr.	
	Control animal	Ascorbic acid deficient 15 days
Cut immediately	52	75
Rinsed in cold saline-buffer: 4–5 minutes	40	55
Stored in saline buffer 2–5 hr.	48	50

60 mgm. liver slices were incubated with 200 μg. cortisone in phosphate-saline buffer for 3 hr. Extraction with chloroform. One-fourth aliquots used for Porter-Silber assay.
Micrograms loss = μg. in prep. initially (200 μg.) − μg. recovered.

5. *Influence of citric acid cycle intermediates in the* in vitro *metabolism of the 17-OH,20,21-ketol structure of cortisone*:

a) Influence on metabolism by liver tissues of control animals: These observations were compiled from studies conducted on days 0, 5, 10, 15, 24, 31, and 36 on the control (pair-feeding) diet. The findings on each individual day were similar; the data on these animals are therefore pooled,

TABLE 3. INFLUENCE OF CITRIC ACID CYCLE INTERMEDIATES ON THE ABILITY OF LIVER SLICES OF CONTROL ANIMALS TO METABOLIZE THE 17-OH,20,21-KETOL STRUCTURE OF CORTISONE

TCA cycle intermediates	n	μg. metabolized per 3 hr.	P (vs. "none")
None	32	52 ± 0.49	
Citrate	22	52 ± 0.76	>0.05
α-Ketoglutarate	20	50 ± 1.52	>0.05
Pyruvate	20	55 ± 1.40	>0.05
Oxalacetate	20	62 ± 1.44	<0.01
Succinate	20	62 ± 1.65	<0.01
Fumarate	20	66 ± 1.20	<0.01
Malate	20	69 ± 1.80	<0.01

Sixty mg. liver slices from control animals (0, 5, 10, 15, 24, 31, and 36 days) were incubated with 200 μg. cortisone in buffer at 37.5° C. for 3 hr. Extraction with chloroform, analysis by Porter-Silber method. TCA cycle intermediates in 0.03 M amounts were used; blanks were negative for Porter-Silber test after extraction and washing procedures.

and the influence of the various Krebs cycle intermediates are considered. The data are presented in Table 3. It is seen that the normal rate of metabolism of the 17-OH,20,21-ketol structure in the presence of the liver slices is 52 ± 0.51 μg./3 hr. when none of the intermediates are added to the preparations. Citrate, pyruvate, of α-ketoglutarate, in 0.03 M amounts failed to alter the metabolism of this structure by these control liver tissues. When succinate, fumarate, malate, or oxalacetate were added to the incubation preparations with control liver tissues, there was noticed significantly increased metabolism of the structure.

b) Influence of citric acid cycle intermediates on the ability of the liver of ascorbic acid-deficient guinea pigs to metabolize the 17-OH,20,21-ketol structure: Since the response of the liver assumed a diphasic pattern, it was thought important to consider the two phases separately. It was observed that the influence of the tricarboxylic acid cycle (TCA cycle) substrates differed during these two periods.

i) First phase of ascorbic acid-deficiency; Animals on the scorbutigenic diet for 5 through 24 days: The liver slices of these animals metabolized

TABLE 4. INFLUENCE OF CITRIC ACID CYCLE INTERMEDIATES ON THE ABILITY OF LIVER SLICES OF ASCORBIC ACID-DEFICIENCY GUINEA PIGS (SCORBUTIGENIC DIET FOR 24 DAYS) TO METABOLIZE THE 17-OH,20-21-KETOL SIDE CHAIN

TCA cycle intermediates	n	μg. metabolized per 3 hr.	P (vs. "None")
None	20	79 ± 0.93	
Citrate	16	53 ± 1.86	<0.01
α-Ketoglutarate	16	55 ± 2.00	<0.01
Oxalacetate	15	64 ± 2.20	<0.01
Pyruvate	15	55 ± 2.80	<0.01
Succinate	15	71 ± 3.34	>0.05
Fumarate	15	76 ± 2.10	>0.05
Malate	10	67 ± 2.70	>0.05

Liver tissue were obtained from guinea pigs on the scorbutigenic diet for 5, 10, 15, 24 days. The responses on the individual days were similar to pooled data presented above.

significantly greater amounts of the side chain structure than did control liver slices. The mean extent of the loss of this structure during the 5–24 day period was 79 ± 0.93 μg. per 60 mg. per 3 hr. This value was derived from pooling of the individual values for the entire 5–24 day period on the scorbutigenic diet. The data are presented in Table 4. It is seen that when pyruvate, citrate, oxalacetate, or α-ketoglutarate, in 0.03 M amounts, are added to incubation preparations, the loss of the structure is decreased significantly (P values are given in the table). The rate reverts to normal levels in the presence of citrate, pyruvate, or α-ketoglutarate. It is seen that oxalacetate is capable of correcting the increased metabolism to a lesser extent than the other three intermediates. Oxalacetate, it is recalled, was capable of increasing the metabolism of the 17-OH,20,21-ketol structure by normal liver tissue (Exp. 5a). In the presence of malate, fumarate,

TABLE 5. INFLUENCE OF CITRIC ACID CYCLE INTERMEDIATES ON THE ABILITY OF LIVER SLICES OF SCORBUTIC GUINEA PIGS (SCORBUTIGENIC DIET 31–36 DAYS) TO METABOLIZE THE 17-OH,20,21-KETOL SIDE CHAIN OF CORTISONE

TCA cycle intermediates	n	μg. metabolized per 3 hr.	P (vs. "None")
None	6	41 ± 2.10	
Citrate	6	37 ± 2.90	>0.05
α-Ketoglutarate	6	42 ± 3.10	>0.05
Pyruvate	6	59 ± 5.60	>0.05
Oxalacetate	6	63 ± 0.70	<0.01
Succinate	6	74 ± 2.70	<0.01
Fumarate	6	73 ± 1.30	<0.01
Malate	6	68 ± 2.10	<0.01

These animals were moribund at the time of the observations. Incubation preparations as described previously.

or succinate the rate of metabolism of the structure by liver tissues of ascorbic-acid-deficient animals (5–24 days) is not altered towards normal.

ii) Moribund animals—days 31 and 36 on the deficient diet: The metabolism of the side chain structure by liver slices of these animals was 41 ± 2.10 μg./60 mg./3 hr. The data on the influence of the TCA cycle intermediates are presented in Table 5. Citrate, pyruvate, and α-ketoglutarate, failed to alter the diminished metabolism of the side chain. On the addition of succinate, malate, fumarate, or oxalacetate, to these liver slice preparations, there was noted increased disappearance of the structure. This action of these metabolites was also observed with preparations from normal animals.

6. *Influence of ascorbic acid (0.03M) on the increased metabolism of the 17-OH,20,21-ketol side chain by liver tissues from deficient animals (5, 10, 15 days)*: The data (Table 6) reveal that ascorbic acid failed to alter the ability of liver tissues of the control animals to metabolize the 17-OH,20,-21-ketol side chain. Similar findings with rat liver tissue were reported earlier (Bacchus, 1953b). On the addition of 0.03 M amounts of sodium ascorbate to the *in vitro* preparations of liver tissue of the 5, 10, and 15 day

TABLE 6. INFLUENCE OF ASCORBIC ACID ON THE ABILITY OF LIVER SLICES TO METABOLIZE THE 17-OH,20,21-KETOL STRUCTURE OF CORTISONE

	Pair-fed Controls (n)	Ascorbic acid-Deficient (5, 10, 15 days) (n)
Ascorbate not added	53 ± 0.70 (17)	78 ± 1.1 (17)
Ascorbate (0.03 M) added to incubation flask	52 ± 2.4 (8)	54 ± 2.5 (8)
P	>0.05	<0.01

Liver slices were obtained from guinea pigs on the scorbutigenic diet for 5, 10, and 15 days, and from appropriate controls pair-fed a complete diet. Ascorbate (0.03 M) as sodium ascorbate was added to the appropriate flasks. Blank preparations, conducted as described in text, revealed negligible Porter-Silber chromogenicity under the conditions employed.

deficient animals (+cortisone) the increased metabolism of the side chain reverted to normal levels. In an earlier study, employing a cruder technique, and a small number of preparations, the conclusion was reached that *in vitro* addition of ascorbate failed to correct the increased metabolism of the side chain by liver tissue of scorbutic animals. The data of the present experiment are highly significant ($P < 0.01$), and in addition, this technique is more reliable.

DISCUSSION

In these experiments we attempted to use the liver slices in the absence of added co-factors in order to determine the presence of underlying differences between the liver tissues of ascorbic acid-deficient and control animals. It was clearly demonstrated that the liver tissue of the early ascorbic acid deficient animals metabolize the 17-OH,20,21-ketol structure of cortisone to a greater extent than normal. The tissues of the moribund, frankly scorbutic, animals metabolize significantly less of this structure. This side chain is representative of the main natural hormonal product in the guinea pig—compound F.

The data demonstrate that the procedure of rinsing, or of storing the tissues in cold buffer markedly influences the ability of the liver tissue to metabolize this structure. It also becomes clear that these procedures obliterate differences between the ascorbic acid-deficient and control animals. The addition of certain citric acid cycle intermediates also obliterates such underlying differences between the liver tissues of these animals.

These data also support the suggestion that data on urinary and plasma corticosteroids should be interpreted in the light of possible changes in the peripheral metabolism before any alterations in adrenal production are claimed. Kayahan (1950) found certain alterations in the urinary corticosteroids and 17-ketosteroids in human subjects given large amounts of ascorbic acid. The changes were ascribed by that author to shifts in the *production* of the adrenal steroids. In our studies we were able to demonstrate similar changes in the character of the urinary steroids in cortisone-treated adrenalectomized female rats given ascorbic acid. Since our data

were collected in adrenalectomized animals, the alterations were not ascribable to shifts in adrenal *production* (Bacchus and Heiffer, 1953b). It is important in studies on adrenal steroids in ascorbic acid deficiency to consider these marked alterations in the ability of the liver to metabolize the 17-OH,20,21-ketol structure. This structure is present in compounds such as cortisone, hydrocortisone, Compound S, and is responsible for the Porter-Silber reaction.

The mechanism of the loss of the 17-OH,20,21-ketol side chain is not fully known. It is known that this structure is at least partially reduced to a glycerol type side-chain (Schneider and Horstmann, 1952; Hechter, Solomon, and Caspi, 1953). It is also probable that certain 17-ketosteroids may be formed as a result of the incubation with liver slices. It has not been possible to detect 17-ketosteroids by the Zimmerman reaction in such preparations (Bacchus, unpublished data; Hechter *et al.*, 1953; Schneider *et al.*, 1952). It has been suggested by Schneider *et al.* (1952) that the failure to find typical ketosteroids might indicate that such compounds might be further metabolized by the *in vitro* system to C 17 hydroxyl group. Interpretation of our data will have to await clarification of the mechanism of the loss of the side chain of corticosteroids.

The exact metabolic lesion responsible for the changes noted in the liver tissues of the ascorbic acid-deficient animals is not clear. Although ascorbic acid added *in vitro* failed to alter the ability of the normal liver to metabolize the side chain, it readily corrected the disturbance in the scorbutic animal, resulting in a normal rate of metabolism of the side-chain. Whether the ability of the vitamin noted here is related to the oxidation-reduction potency of the system ascorbic acid⇆dehydroascorbic acid requires further investigation.

The experiments with the tricarboxylic acid cycle intermediates demonstrate some well-marked patterns. The C_4 dicarboxylic acids malate, fumarate, and succinate, were each capable of increasing the loss of the 17-OH,20,21-ketol side chain in the presence of normal liver tissues. Citrate, pyruvate, and α-ketoglutarate did not exhibit this ability. It is recalled that Dorfman, Hayano, Savard, and Haynes (1953) found that these dicarboxylic acids, malate, succinate, and fumarate, were capable of enhancing the conversion of 11-desoxycorticosterone to corticosterone, in preparations of adrenal homogenates. Citrate, oxalacetate, α-ketoglutarate, and pyruvate, were ineffective. In our experiments with liver tissues it is seen that citrate, α-ketoglutarate, pyruvate, and oxalacetate, are capable of normalizing the liver of the scorbutic animal, insofar as the metabolism of the 17-OH,20,21-ketol side chain is involved. Whether there is a deficiency of these substances in ascorbic acid deficiency is not known. It has been demonstrated, however, that the process of glycolysis is diminished in ascorbic acid-deficiency (see data reviewed by Pirani (1952).

It is hoped that studies on the metabolites from these *in vitro* prepara-

tions will permit elucidation of the mechanism of the loss of the side chain. It might then be possible to fully interpret the role of the Krebs cycle intermediates in this process.

SUMMARY

The *in vitro* metabolism of the 17-OH,20,21-ketol side chain of cortisone by liver slices was investigated. Liver tissues were obtained from guinea pigs on a scorbutigenic diet, and from pair-fed control, and normal, guinea pigs. The normal and control liver metabolizes the structure to the extent of 52 μg. per 60 mg. per 3 hr. There was a diphasic pattern in the ability of the guinea pig on the scorbutigenic fare to metabolize the side chain. Up to 24 days on the deficient fare the liver tissues metabolize significantly greater amounts of the structure. After 31–36 days on the scorbutigenic fare, when the animals were moribund, there was a decrease in the ability of the liver to metabolize this structure.

Pyruvate, oxalacetate, α-ketoglutarate, citrate, and ascorbate, in 0.03 M amounts, were each capable of preventing the increased metabolism of the side chain by the liver tissue of the scorbutic animal. The substrates citrate, α-ketoglutarate, pyruvate, and ascorbate, each failed to alter the metabolism of the side chain by liver tissues of normal animals. Oxalacetate, succinate, fumarate, and malate, on addition to preparations of normal liver tissue slices, were each capable of increasing the metabolism of the side chain of cortisone. These metabolites, with the exception of oxalacetate, failed to alter the ability of the liver of the ascorbic acid-deficient (5–24 days) animal to metabolize increased amounts of the side chain.

These data are discussed in relation to the importance of evaluating the peripheral metabolism of corticosteroids before plasma and urine levels are employed as reliable indices of adrenal activity. It was also pointed out that it is important to use the liver in fresh unwashed state in order to detect underlying differences between liver tissues of ascorbic acid-deficient, and control, animals.

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THE LOSS OF ESTROGEN-PROTEIN BINDING CAPACITY IN RAT HEPATOMA[1]

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PREVIOUS investigations from this laboratory have revealed the presence of enzymic mechanisms in rat liver which promote the binding *in vitro* of isotopically-labeled estrone or its metabolites to the proteins of the serum medium as well as to those of the surviving tissue (Szego, 1953). One aspect of these findings has been confirmed by Riegel and Mueller (1954) who observed incorporation of radioactivity derived from estradiol-16-C^{14} into the "insoluble" proteins of liver homogenates.

The present investigation deals with the measurement of estrogen-protein binding *in vitro* in primary and transplanted hepatoma. The results indicate that the binding of estrone or its metabolites to the proteins of the serum medium is greatly reduced in the presence of the pathological tissues. Additional aberrations of estrone metabolism in the neoplastic tissues are described.

MATERIALS AND METHODS

The procedures used in the present study, with exceptions and additions specifically noted below, have been described in detail elsewhere (Szego, 1953).

The incubation studies were conducted in freshly-obtained rat serum containing estrone-16-C^{14},[2] as previously described. The animals supplying the serum for these studies were female Sprague-Dawley or Irish rats, 4 to 6 months old, maintained on Rockland laboratory chow with a supplement of lettuce twice weekly. Pooled homologous serum was obtained by exsanguination from the dorsal aorta under light Nembutal anaesthesia. Normal control liver tissue was provided by untreated Sprague-Dawley and Irish rats, respectively. In general, incubation was carried out for 2 hours in 20 ml. beakers under a constant stream of 95% O_2—5% CO_2 in a Dubnoff metabolic incubator at 37.5 to 38° C. In view of the need for maintaining the pCO_2, and thus, the normal pH of the serum medium during incubation, CO_2 collection for subsequent radioactivity measurements was not feasible. Except as otherwise noted, each beaker contained 5.82 μg. estrone-

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[2] The isotopic estrone used in most of the experiments was generously provided by Prof. R. D. H. Heard. A few of the studies were conducted with similar material obtained from the Charles E. Frosst Co., Montreal.

16-C^{14} in 3 ml. serum medium, in the presence of <1% ethanol. Tissues were prepared by freehand mincing with a single-edged razor blade on a clean cork with a large surface. Each incubation vessel contained approximately 150 mg. of tissue. Representative aliquots of liver were taken for semi-micro Kjeldahl analysis. A portion of each liver was preserved for histological section[3] to permit diagnosis of the tumorous, precancerous, or cirrhotic condition of the tissue.[4]

Primary hepatoma were induced in Sprague-Dawley females by the *ad libitum* feeding of a low protein diet[5] containing 0.06% by weight of Merck reagent grade *p*-dimethylaminoazobenzene (DAB). Controls for these animals were maintained on the same regime without DAB. Body weight gain on these diets was not significantly different in the two groups except for a short initial decline in the DAB series. However, growth was reduced with respect to normal controls. Animals on these regimes, with or without tumors, were sacrificed at intervals and provided liver tissue for the incubation experiments. The age at sacrifice ranged from 7.5 to 8 months. Transplanted hepatoma originally induced in the Sprague-Dawley strain by DAB were also employed. These were first-generation transplants of 6 weeks' duration, and were prepared by direct trochar implantation into the recipient liver under light ether anaesthesia. Bleeding was controlled by fibrin foam. All tumor animals were in good condition at time of sacrifice and the tumors did not appear necrotic.

The transplantable hepatoma carried in the Irish rats[6] was used 11 days after implantation. Transplantation of this tumor was accomplished through intraperitoneal inoculation with 0.5 ml. of a 25% (wet wt.: vol.) suspension of tumor obtained by sterile procedures. The tumors used in the present study were in the 21st transplanted generation since receipt from Dr. Harold P. Morris of the National Cancer Institute.

At the end of the incubation period the vessels were rapidly chilled and their contents centrifuged in the cold as previously described (Szego, 1953). The proteins of the decanted serum were then immediately precipitated by dropwise addition to 150 ml. acetone at 0° C. with mechanical stirring. All samples were taken to this stage without delay.

Extraction of the resulting serum supernate, serum proteins, and the tissue residue remaining in the centrifuge tube was carried out as detailed earlier (Szego, 1953), with the exceptions noted below. Designation of the fractions in the present report by letters (A) through (H) is in accordance with the notations earlier established (Szego, 1953). The exceptions to the previously-described fractionation scheme are as follows:

1. The serum supernates in the present study were not subjected to acid hydrolysis as in the earlier study (Szego, 1953), but were extracted with ether directly after removal

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[3] Miss Alice Rudolph kindly prepared the histological specimens.

[4] The writer is indebted to Dr. R. Kinosita for diagnosis of the pathological preparations and for many helpful suggestions.

[5] The low protein diet referred to in the text and Tables was similar to a modified Steenbock mixture (cf. Bills *et al.*, 1931), but "diluted" 1:1 by weight with potato flour to establish nutritional deficiencies conducive to relatively rapid tumor induction (Kinosita, 1937; György, Poling and Goldblatt, 1941; Kensler *et al.*, 1941; Miller *et al.*, 1941). DAB (see text), dissolved in 100 ml. warmed olive oil per 5 Kg. of the above mixture, was added to a final concentration of 0.06% by weight. The low-protein control diet contained the equivalent amount of olive oil without DAB. Both diets were stored in the refrigerator. They were fed *ad lib.* Animals on these diets did not receive the supplement of lettuce fed the normal controls.

[6] Obtained through the courtesy of Dr. A. M. Schechtman and Mr. P. Terasaki. No. 3683 in *Survey of Transplantable and Transmissible Animal Tumors* (Dunham and Stewart, 1953).

of the last traces of acetone, dilution to 100 ml. with water, and adjustment to pH 4. This modification was based on recovery studies which indicated that negligible ether-soluble radioactivity was present in this fraction following acid hydrolysis of the aqueous residue after direct ether extraction. Thus, the supernate ether extract A in the present report represents extraction of unhydrolyzed material, unlike the fraction previously (Szego, 1953) designated by the same letter. The water back-wash fraction of the present fraction A, also containing minimal amounts of radioactivity (cf. Szego, 1953), was not saved for analysis in the present study, hence no fraction B is reported in the current series.

2. The liver residues in the present report were treated in the same manner as previously described except that in most instances they were stored in the deep freeze for variable periods before fractionation according to Fig. 1B in the previous report (Szego, 1953). The results were comparable to the earlier findings. An additional departure from the previously-described fractionation scheme was that in a few instances an aliquot of the aqueous liver residue G was plated without concentration by lyophilization. No appreciable influence on the results was observed as a result of these minor variations.

Radioactivity measurements were made in a windowless flow counter, and corrections for self-absorption applied as previously described (Szego, 1953).

RESULTS AND DISCUSSION

The recovery of radioactivity in various fractions of the incubation medium and of the surviving tissues is shown in Table 1. Over-all recovery of total radioactivity in the normal samples was of the same order of magnitude as earlier observed (Szego, 1953). The ether-soluble radioactivity released by vigorous acid hydrolysis from the proteins of the serum medium incubated in the presence of normal liver (fraction D) averaged 5.82% of the radioactivity originally added. Appreciable estrogenic activity was detected in this fraction. It will be observed from Table 1 that serum protein-bound isotopic activity in parallel samples incubated in the presence of primary hepatoma induced by DAB was negligible. The control on the low protein diet exhibited neither significant liver pathology (beyond a slight increase in histologically-demonstrable lipide), nor any curtailment in capacity to promote protein-binding of isotope at a time when hepatoma had already developed in the DAB group. However, in those animals in which cirrhosis[4] developed on the low protein diet with or without DAB, some reduction in capacity to promote the binding of estrone or its metabolites to the serum proteins was evident, well correlated with the extent of liver pathology. This reduction was particularly striking in the animals on the DAB diet at a time when the lesions appeared precancerous.[4] Thus, in three animals maintained for 133 days on the DAB diet, the percentage of added C^{14} recovered in fraction D averaged 1.73. A corresponding decrease was noted in fraction E (cf. below). Further decline was apparent in the fully developed tumors (cf. Table 1).[7]

[7] While emphasis has been placed herein on progressive decline with tumor induction of hepatic capacity to promote the protein binding of estrone or its metabolites, it should be pointed out that this enzymic activity was sharply augmented during the early stages

TABLE 1. THE INFLUENCE OF SURVIVING LIVER TISSUE ON THE DISTRIBUTION* OF RADIOACTIVITY FOLLOWING INCUBATION WITH ESTRONE-16-C^{14} IN HOMOLOGOUS SERUM MEDIUM (SPRAGUE-DAWLEY ♀)

Serum supernate	Serum proteins				Liver				Grand total
Ether extract (A)	Ether soluble (mild hydrolysis) (C)	Ether soluble (strong hydrolysis) (D)	H_2O backwash (E)	Total	Ether soluble (mild hydrolysis) (F)	Aqueous residue (G)	Ether soluble (strong hydrolysis) (H)	"Total"[1] (F+G)	
Serum Control[2]									
91.0	0.63	0.08	0	0.71	—	—	—	—	91.71
Normal Liver									
51.5	1.03	4.33	2.36	7.72	2.28	9.42	1.79	11.70	70.92
53.0	0.69	5.05	5.77	11.51	3.18	8.57	1.50	11.75	76.26
71.0	0.50	5.33	6.16	11.99	2.78	10.30	1.81	13.08	96.07
64.9	0.80	6.31	8.44	15.55	2.30	8.26	1.56	10.56	91.01
{60.3	0.54	5.35	—	>5.89	2.16	8.05	1.33	10.21	>76.40
{62.2	0.59	4.87	—	>5.46	3.48	8.20	1.52	11.68	>79.34
47.5	0.86	6.40	5.95	13.21	4.24	10.60	1.92	14.84	75.55
64.0[3]	1.00	8.90	—	>9.90	4.47	10.80	1.98	15.27	>89.20
Low Protein Diet, (143 days)[4]									
63.8	0.84	6.50	5.65	12.99	2.53	13.40	1.51	15.93	92.72
Primary Hepatoma[5]									
108.0	0.67	0.18	0	0.85	5.61	3.59	0.52	9.20	118.05
{108.0[6]	0.46	0.35	0.81	1.62	3.08	4.14	0.64	7.22	116.84
{108.0[6]	0.45	0.45	0.23	1.13	5.00	4.67	0.94	9.67	118.80
Transplanted Hepatoma[7]									
{101.0	0.63	0.12	0.10	0.85	4.90	2.45	0.36	7.35	109.20
{ 79.5	0.63	0.08	0	0.71	5.79	2.65	0.20	8.44	88.65

* For fraction designated see text and Szego (1953). Values indicated are % added C^{14} recovered (of 10,200 cpm ≅5.82 μg. added per 3 ml. total medium at 0 time, unless otherwise indicated). All samples incubated 2 hrs. Where successive samples are enclosed in brackets, in this or subsequent Tables, they represent duplicate incubates of the same tissue.

[1] "Total" in liver represents, in part, adsorbed material from serum medium (present in ether-soluble fraction after mild alkaline hydrolysis. Cf. Szego, 1953).

[2] Incubated in the absence of added tissue.

[3] Frosst estrone, 12,100 cpm ≅5.82 μg. added per 3 ml. in this case.

[4] Control for first animal of primary hepatoma group. The latter was sacrificed after 143 days on the same diet containing 0.06% *p*-dimethylaminoazobenzene (DAB). See text.

[5] Histological sections of this group of tissues revealed mixed adenocarcinoma and cholangioma.

[6] On DAB diet for 165 days.

[7] Adenocarcinoma uncomplicated by cholangioma. Hemorrhagic areas typical of this tumor were evident.

It is further clear from Table 1 that this enzymic capacity is virtually absent from transplanted hepatoma tissue of the Sprague-Dawley strain. Under these conditions, the effects of dietary restriction are obviated. Table 2 likewise reveals a defect in the capacity of transplanted hepatoma of the Irish strain to promote the binding of estrone-16-C^{14} or its isotopic metabolites to the proteins of the serum incubation medium (fraction D). It will be observed that samples of grossly uninvolved liver tissue from the hepatoma donor animal were intermediate in activity between control and hepatoma material in this regard.

(4–54 days) of dietary injury, especially in the DAB group, to beyond the range previously reported for liver tissue regenerating after partial hepatectomy (Szego, 1953). Under these circumstances, the reparative process prevails, as evidenced by histological sections.[4]

TABLE 2. THE INFLUENCE OF SURVIVING LIVER TISSUE ON THE DISTRIBUTION* OF RADIOACTIVITY FOLLOWING INCUBATION WITH ESTRONE-16-C^{14} IN HOMOLOGOUS SERUM MEDIUM (IRISH STRAIN)

Serum supernate	Serum proteins				Liver				Grand Total
Ether extract (A)	Ether soluble (mild hydrolysis) (C)	Ether soluble (strong hydrolysis) (D)	H_2O back-wash (E)	Total	Ether soluble (mild hydrolysis) (F)	Aqueous residue (G)	Ether soluble (strong hydrolysis) (H)	"Total"[1] (F+G)	
Normal Liver									
♂ 39.4	2.61	5.67	5.07	13.35	1.79	6.38	1.77	8.17	60.92
♀ 49.0	1.15	7.43	5.91	14.49	3.61	9.22	2.32	12.83	76.32
Transplanted Hepatoma, ♂									
93.0	0.48	1.00	2.23	3.71	3.75	3.44	0.52	7.19	103.90
86.3	0.86	0.96	1.21	3.03	5.91	2.16	0.42	8.07	97.40
Uninvolved Liver, ♂[2]									
55.3	2.47	3.25	2.66	8.38	2.69	6.38	1.32	9.07	72.75
50.0	2.26	3.30	2.03	7.59	4.14	6.76	1.31	10.90	68.49

* As in Table 1.
[1] As in Table 1.
[2] From hepatoma donor.

In contrast to the above results, the adsorption (rather than binding) of the radioactive estrogen or its metabolites to the serum proteins in the presence of surviving liver was independent of the physiological state of the tissue. Fraction C represents ether-soluble material extracted following mild alkaline digestion of the serum proteins of the incubate, and radioactivity therein is apparently due to nonspecific adsorption (cf., Table 1, serum control). Evidence in support of this contention was also presented in an earlier report (Szego, 1953).

In general, the activity of fraction E, the water back-wash of the material which is released in ether-soluble form after strong acid hydrolysis of the serum proteins, paralleled the values observed in the latter (D). This had been noted in the earlier report (Szego, 1953) and is interpreted as suggestive either of incomplete hydrolysis of conjugated material or of extensive degradation of the estrone molecule. It will be noted in the Tables that the level of activity observed in this fraction following incubation of the neoplastic material is in all cases lower than that of the appropriate controls. This is suggestive of further deficiency in the capacity of neoplastic liver to metabolize the estrogen.

Incorporation of isotope derived from estrone-16-C^{14} into liver fractions G and H was similarly depressed in the hepatomatous tissues (cf. Tables 1, 2), but not in precancerous[4] specimens or low-protein dietary controls. Fraction F (ether-soluble material following mild alkaline digestion of the centrifuged but unwashed tissue residue after incubation), is not considered significant except to aid in accounting for over-all total radioactivity recovered from the entire sample for reasons which are indicated in footnote 1 to the Tables (cf., also Szego, 1953). The lack of specificity of liver frac-

tion F is further borne out by its independence of the character of the tissue incubated. Fraction G, however (aqueous residue of the digested liver tissue after removal of ether-soluble material F), showed significantly less incorporation of isotope when incubation had been carried out in the presence of primary Sprague-Dawley hepatoma, or the transplanted tumor of either strain. The uninvolved liver of the Irish strain incorporated levels of isotope into fraction G which were not significantly different from the appropriate control, although its *serum* protein-binding capacity (fraction D; cf., Table 2) was markedly reduced. The bulk of the isotopic material in liver fraction G is non-estrogenic and, in fact, non-lipoidal since a mere fraction of its initial isotope content could be released in ether-soluble form by strong acid hydrolysis (cf., also Szego, 1953). Analogous results were observed by Riegel and Mueller (1954). In keeping with the reduced level of isotope seen in liver fraction G when incubation was carried out in the presence of hepatoma of both strains, fraction H was correspondingly lower (Tables 1, 2).

In harmony with the apparent failure of hepatoma to metabolize estrone by all routes to the degree seen in the controls, it will be observed that the amount of ether-soluble radioactivity recovered from the supernate, probably representative of unmetabolized substrate, is greater in the neoplastic than in the control incubates. The over-all recovery from all fractions is similarly of a higher order of magnitude in the pathological specimens (cf. Tables 1, 2). A trend toward reduced metabolism of estrogen incubated in the presence of surviving hepatoma may be observed in the recent report of Ryan and Engel (1953), but is inconclusive due to excessive variability and limited numbers of observations. If collection of CO_2 liberated during incubation had been feasible in the present study, determination of its isotopic content might have been helpful in accounting for the small but consistent deficit in total radioactivity recovered in the normal samples.

The cause of the multiple defects in estrone metabolism exhibited by the neoplastic tissues under the conditions herein described has not been determined. The nitrogen content of the hepatoma samples was significantly lower than that of the control livers in all cases (cf., also Mills and Smith, 1951), and consequently the amount of tissue (enzyme) protein present *a priori* in the approximately 150 mg. weighed into the incubation vessels, was somewhat lower in the case of the tumors. However, the order of magnitude of this difference (18% in the Sprague-Dawley tumors and 10% in those of the Irish strain) was insufficient to account for the degree of diminution of estrogen-protein binding capacity exhibited by these tissues (94–98%, and 83%, respectively). Qualtitative or quantitative differences in cellular protein and enzymic composition through "crowding-out" of normal components appears possible.

A striking parallel to the apparent loss of estrogen-protein binding capacity by the tumors may be found in the observations of Miller and

Miller (1947) who noted that the property of firm binding of the azo dye DAB by normal rat liver was lacking in the neoplasm. These workers postulated that certain protein constituents responsible for this phenomenon in normal liver were lost or altered in the process of tumor development (Sorof *et al.* 1951a, b; Hoffman and Schechtman, 1952). A similar situation may exist with respect to the hepatic enzyme involved in estrogen-protein binding.

The specific function of the liver in promoting estrogen-protein binding appears to involve the formation of the glucuronide or some other water-soluble conjugate of the steroid, intermediate to its association with a specific serum protein carrier (Roberts and Szego, 1946, 1953, 1955; Szego, 1953; Szego and Roberts, 1953; Szego and Wolcott, 1955). While glucuronide synthesis has not been determined, sulfate conjugating capacity of high-speed supernates of a typical DAB hepatoma, as measured with the synthetic substrate *m*-aminophenol (Segal, 1955), was found in these laboratories[8] to be negligible as compared to normal liver. Mills and Smith (1951) had noted that the hydrolytic enzyme β-glucuronidase was decreased in DAB-induced hepatoma, both on the basis of total activity and in terms of DNA content. Catalase activity measured[9] on aliquots of the same tissues used for the experiment on the Irish strain (Table 2) was significantly depressed in the transplanted hepatoma (Dagg, 1953). These observations are in line with growing evidence for the loss of specific enzymic mechanisms in chemically induced hepatoma (Greenstein, 1946, 1954). It is of interest to note that the estrogen-metabolizing enzymic activity of the transplanted hepatoma samples, as reflected by the isotope content of fractions D, E, G, and H, appeared even more drastically curtailed than that of the primary hepatoma series (Table 1). This is in accord with the observations of Greenstein and others (Greenstein, 1946) that a portion of those enzyme systems which are altered from normal in primary hepatoma undergoes further alteration in the same direction in the course of serial transplantation.

It is also possible that certain co-factors essential for estrogen-protein binding may be lacking in hepatomatous tissue. One aspect of this problem was investigated by fortification of the medium with DPN. Enhancement in serum protein fraction D was observed in both normal and neoplastic tissue incubates in the presence of DPN (Table 3), but restoration of the latter tissues to the capacity of the normal controls was not accomplished. DPN fortification had no consistent effect on incorporation of isotope into liver fractions. That the small effects of DPN which were seen in the present study were probably valid is supported by the slightly de-

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[8] Thanks are due to Dr. Harold L. Segal for kindly performing this determination.

[9] The writer is indebted to Dr. C. P. Dagg for permission to cite these unpublished observations.

TABLE 3. THE INFLUENCE OF DPN AND NICOTINAMIDE ON THE DISTRIBUTION* OF RADIOACTIVITY FOLLOWING INCUBATION WITH ESTRONE-16-C^{14} IN THE PRESENCE OF NORMAL LIVER AND PRIMARY HEPATOMA (SPRAGUE-DAWLEY ♀)

Additive	Serum supernate	Serum proteins				Liver				Grand Total
	Ether extract (A)	Ether soluble (mild hydrolysis) (C)	Ether soluble (strong hydrolysis) (D)	H_2O backwash (E)	Total	Ether soluble (mild hydrolysis) (F)	Aqueous residue (G)	Ether soluble (strong hydrolysis) (H)	"Total"[1] (F+G)	
Normal Liver										
—	64.0	1.00	8.90	—	>9.90	4.47	10.80	1.98	15.27	>89.17
DPN[2]	—	1.28	11.60	13.70	26.58	2.78	10.00	1.72	12.78	—
Primary Hepatoma[3]										
—	108.0	0.46	0.35	0.81	1.62	3.08	4.14	0.64	7.22	116.84
—	108.0	0.45	0.45	0.23	1.13	5.00	4.67	0.94	9.67	118.80
DPN[2]	94.5	0.53	0.86	1.57	2.96	6.35	2.54	0.52	8.89	106.35
DPN[2]	96.4	0.79	1.93	0.53	3.25	3.79	5.62	0.65	9.41	109.06

* As in Table 1 with the exception that 12,100 cpm ≅5.82 μg. estrone-16-C^{14} (Frosst) added per 3 ml. total medium at zero time.
[1] As in Table 1.
[2] Where DPN is indicated, the serum medium was fortified to the following final concentrations: DPN (Pabst), 10^{-3}M; nicotinamide, 2×10^{-2}M; $MgSO_4$, 10^{-3}M.
[3] On DAB diet for 165 days.

creased amount of unmetabolized radioactivity remaining in the supernates of the DPN-fortified hepatoma samples, coupled with the decrease in over-all activity accounted for in all fractions. Similar data have been cited above as indirect evidence of extent of metabolic influence exerted by the liver tissues. Coppedge *et al.* (1948) and De Meio and co-workers (1948), have demonstrated the participation of a dehydrogenase system requiring DPN in the hepatic inactivation of estradiol under conditions considerably different from those used in the present investigation. However, on the basis of the data in the present report, it appears that a more fundamental defect than lack of DPN is responsible for the inefficiency of the hepatoma with respect to estrone metabolism. The basic protein alterations discussed above would appear more cogent.

SUMMARY

Studies of an enzyme system in rat liver capable of promoting the binding of estrone-16-C^{14} or its isotopic metabolites to the proteins of the homologous serum incubation medium have been extended. Primary and transplanted hepatomatous tissues were shown to be defective in this regard. Additional aberrations of estrone metabolism were demonstrated in the presence of the neoplastic tissues. Recovery of radioisotope bound to the liver proteins was also significantly and consistently depressed. In addition, evidence was obtained that the tumor tissue was less effective than normal liver in transforming the steroid to unidentified metabolites. The possible relationship of these findings to known alterations in liver composition during tumorigenesis has been discussed.

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EFFECT OF PROLONGED PRETREATMENT WITH DESOXYCORTICOSTERONE ON THE LIVER OF HEPATECTOMIZED RATS[1]

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LIVER regeneration in partially hepatectomized rats is significantly influenced by adrenalectomy with or without treatment with adrenal-cortical hormones. Adrenalectomy impairs the capacity of the liver to regenerate, but this capacity is restored to it by treatment with adrenal-cortical extracts (Berman *et al.*, 1947, Friedgood *et al.*, 1950, Uyldert, 1950). Desoxycorticosterone acetate (DCA) was found to promote liver regeneration in adrenalectomized and intact rats (Berman *et al.*, 1947, Friedgood *et al.*, 1950, Fanfani and Marconi, 1951, Uyldert, 1950), while cortisone inhibited multiplication of liver cells following partial hepatectomy (Einhorn *et al.*, 1954). The rate of early regeneration immediately after hepatectomy was found to be greatest in rats treated with adrenocorticotrophic hormone (ACTH) (Roberts, 1951). This relationship between the adrenal glands and regenerative capacity of the liver is interesting in view of the fact that adrenal glands enhance carcinogenesis of the liver of rats fed para-dimethylaminoazobenzene (Symeonidis *et al.*, 1954). The present experiments deal chiefly with the effect on the regenerative capacity of the liver, of prolonged continuous treatment with desoxycorticosterone acetate (DCA) for 6 months prior to hepatectomy. In all previous studies, by other investigators, rats were treated with DCA only during the time of regeneration of the liver.

MATERIAL AND METHODS

Thirty-seven male Osborne-Mendel rats, 9 to 12 weeks of age (mean weight 255 gm.) were divided into two groups: 1. Eighteen rats were treated with desoxycorticosterone acetate for 6 months; and 2. Nineteen rats were left untreated with DCA. A pellet of DCA contained 16 mg. of pure crystalline desoxycorticosterone acetate and was implanted subcutaneously in the back with a trocar. Each of the treated rats received a set of 4

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pellets every four weeks, until a total of six such sets (384 mg. DCA) were implanted in him. All of the pellets were left in the animals until the time of autopsy when the unabsorbed remnants were dissected from the subcutaneous tissue, desiccated and weighed. The rats had free access to food (Derwood rat food pellets) and drinking water.

All the rats in both the groups were partially hepatectomized at 28 weeks after the first set of desoxycorticosterone pellets was implanted in rats of the first group. At the time of hepatectomy, the animals were about 38 weeks old, with a mean weight of 389 gm. Partial hepatectomy was performed according to the method of Brues *et al.* (1938). Under ether anesthesia, the left lateral and medial lobes were excised. The branches of the portal vein were ligated prior to ligation of the hepatic veins in order to facilitate exsanguination of the lobes to be excised.

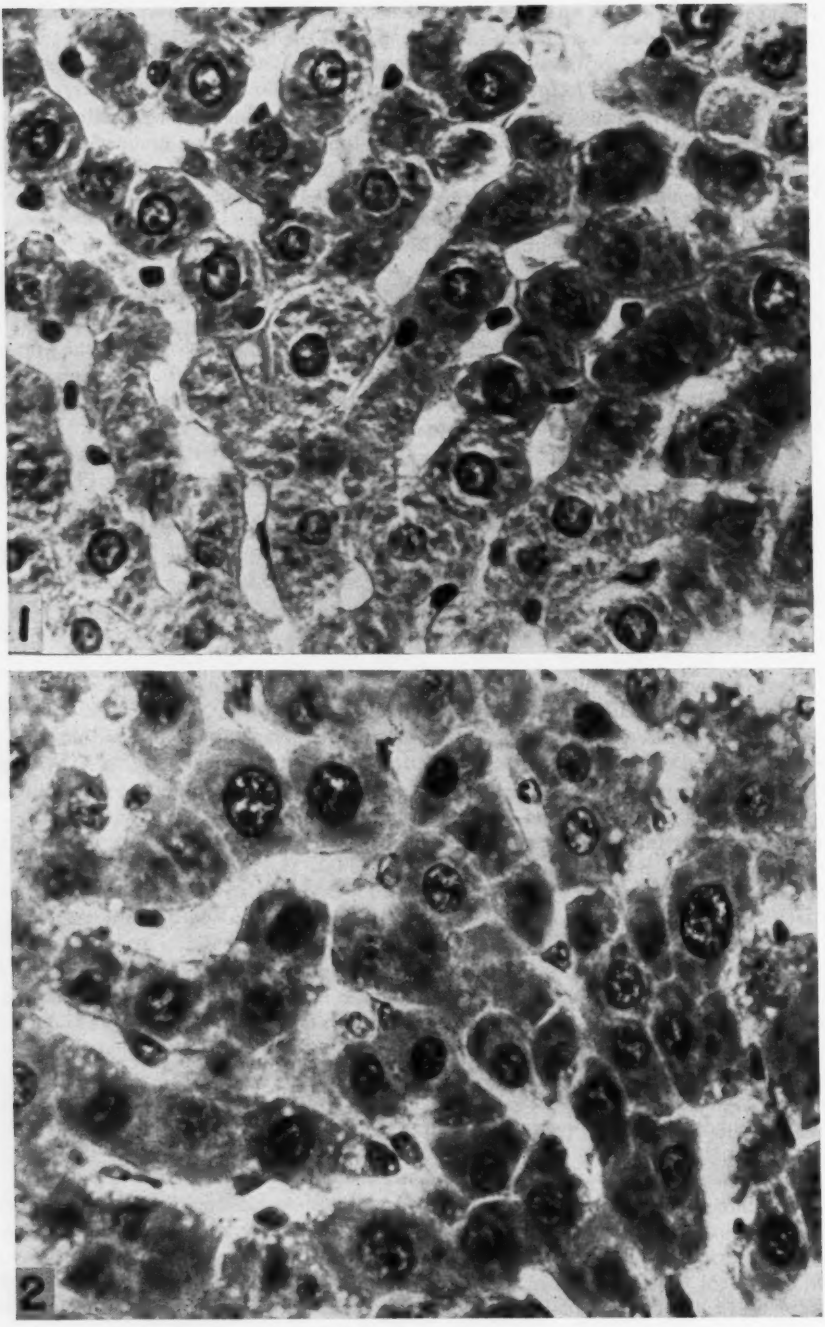
The rats were killed by exsanguination under ether anesthesia on the 0, 1st, 3rd, 7th, and 14th day after the operation. The animals were starved for 24 hours before operation and before autopsy. The rats and excised lobes of their livers were weighed at the time of hepatectomy. Complete autopsies were performed. The weight of the liver and of the adrenal glands was recorded. Portion of the operatively excised liver lobes and of the liver excised at autopsy was used for chemical analysis.

Six rats from each group (DCA-treated and untreated) were killed on day zero, right after the partial hepatectomy was performed. The lobes of their livers removed by partial hepatectomy, and the lobes remaining after hepatectomy were weighed separately. These two weights of the liver portions added together constituted the total weight of the liver at the time of hepatectomy. The excised median and left lateral lobes of the rats after 28 weeks of treatment with desoxycorticosterone acetate, were found to constitute 71.0 ± 2.26 % of the total weight of the liver tissue. In the untreated rats, these lobes comprised 70.8 ± 1.92% of the total liver tissue. These values are fairly close to the corresponding figure (68.4%) of Brues *et al.* (1938). The ratio of the weight of excised lobes to that of the lobes left intact was sufficiently constant in all the specimens in both the groups to permit calculation of total liver weight from the weight of the excised lobes of the partially hepatectomized animals. These estimated liver weights are used in all calculations based on total liver weight at the time of hepatectomy.

All liver specimens for chemical analysis, as soon as removed from the animals, were placed in weighing bottles and stored in the deep freeze, until the time of analysis. Analytical procedures were started on every specimen within 24 hours after its removal from the animal. Every liver specimen was divided into two parts. The part for moisture and fat determination was weighed and placed for drying in an oven at 90° C. Another part for protein and non-protein nitrogen determination was weighed and homogenized in a glass homogenizer.

The sample for moisture and fat determination was dried to a constant weight, and the moisture content calculated from the difference between fresh and dry weight. The dry sample was ground to a fine powder in a mortar and a weighed sample was refluxed for 2 hours with a mixture (50 ml. per gram of sample) of 3 parts of 95% ethanol and one of ethyl ether. The alcohol-ether extract was separated from the residue and the process repeated two more times. The combined alcohol-ether extract was evaporated to dryness at 70° C. under reduced pressure and the residue extracted with 30 ml. of boiling petroleum ether three times. Combined petroleum-ether extract was collected in a weighing bottle, evaporated just to dryness, and weighed as crude fat.

The homogenized sample for protein and non-protein nitrogen determination was suspended in enough water to make 10 ml. of suspension per gram of material. The proteins were precipitated by adding enough 40% trichloracetic acid to the suspension to bring its acid concentration up to 10%. Nonprotein nitrogen was determined on the supernatant by micro-Kjeldahl digestion, Nesslerization, and colorimetry, and protein



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nitrogen was determined on the precipitate by Kjeldahl method of digestion, distillation and titration.

Specimens for histologic study were fixed in Zenker's acetic fluid, embedded in paraffin and stained with hematoxylin and eosin. The specimens were also treated by the periodic acid-Schiff technique and the Wilder method for reticulum. Formalin-fixed material was used in staining for lipoid substances.

The excised and regenerated livers were studied histologically. The following units were quantitatively determined by the method of Chalkley (1943): liver cells, multinucleated liver cells, liver-cell nucleoli, liver cells in mitosis, bile-duct cells, Kuppfer cells and liver sinusoids. A total of 600 units were counted in each liver. Analysis of variance was employed for the statistical[4] evaluation of the findings.

RESULTS

Desoxycorticosterone acetate absorption during the experimental period of 30 weeks (28 before and 2 after partial hepatectomy) from implanted DCA pellets averaged 1.1 mg. per day per rat.

Changes in the liver: Livers of DCA treated rats were significantly[5] larger than those of the untreated rats on day 0 (Table 1), and on subsequent days after liver regeneration. The body weights of the two groups of animals were about the same, being 397 gm. and 381 gm., respectively. The differences in total-liver weight (11.40 gm.) and of the excised lobes (8.41 gm.) in DCA-treated rats compared with the untreated rats (9.02

TABLE 1. WEIGHT OF THE ANIMAL AND WEIGHT OF THE LIVER AND ITS CHEMICAL ANALYSIS, BEFORE AND AT STATED INTERVALS AFTER PARTIAL HEPATECTOMY

Time after operation in days	Number of rats	Animal weight at operation (gm.)		Animal weight at autopsy (gm.)		Liver weight (gm.)		Liver weight per 100 gm. body weight (gm.)		Fat mg. per gm. fresh liver		N.P.N. mgs. per gm. fresh liver		Protein N. mg. per gm. fresh liver		Liver Protein N. mg. per animal		Liver moisture as percentage of fresh weight	
		C	DCA	C	DCA	C	DCA	C	DCA	C	DCA	C	DCA	C	DCA	C	DCA	C	DCA
0*	12			363	377	9.0	11.4	2.5	3.0	60	58	3.1	2.8	32.2	30.7	290	350	66	67
0†	25	397	381			7.4	8.4	1.9	2.2	00	54	2.7	2.9	29.8	29.1	221	244	67	68
1	6	387	369	365	349	4.8	4.9	1.3	1.3	—	—	2.5	3.4	18.7	21.3	90	102	66	69
3	6	411	382	374	353	7.0	6.8	1.8	1.9	65	57	2.8	3.6	29.3	31.0	202	211	70	69
7	7	376	387	344	351	7.7	8.7	2.2	2.4	52	52	3.1	3.5	31.8	31.4	244	273	68	70
14	6	412	393	399	380	8.2	8.4	2.1	2.5	59	64	3.1	2.3	31.3	26.6	256	255	68	67

* Rats killed immediately after operation.
† Excised lobes.
C = Rats not treated with DCA.
DCA = Animals with implants of desoxycorticosterone acetate.

[4] For the statistical analysis we are indebted to Mr. Marvin Schneiderman of the Biometrics Section, National Cancer Institute.

[5] Every significant difference had a P value of less than 0.01.

FIG. 1. Liver of a rat on the day (zero day) of partial hepatectomy. Size and shape of the liver cells are normal. Nuclei usually contain one nucleolus per nucleus. They are uniform in size and shape. Hematoxylin and eosin. ×800.

FIG. 2. Regenerated liver of rat three days after partial hepatectomy. Liver cells are enlarged with vacuolated cytoplasm (fat droplets). Nuclei are large and pleomorphic. They usually contain more than one nucleolus. Hematoxylin and eosin. ×800.

TABLE 2. LIVER REGENERATION INDEX. WEIGHT OF REGENERATING LIVER AS A PERCENTAGE OF PRE-HEPATECTOMY LIVER WEIGHT

	Days after hepatectomy				
	0	1	3	7	14
Rats treated with desoxycorticosterone acetate	29*	40	56	76	81
Rats not treated with desoxycorticosterone acetate	29*	48	67	77	74

* Portion of liver remaining, immediately after partial hepatectomy.

gm. for the total liver and 7.39 gm. for the excised lobes) is significant. The weight ratio of remaining lobes to that of the excised lobes was 0.4102 as determined for rats at 0 day. The pattern of regeneration in the liver in DCA and untreated animals according to net liver growth after partial hepatectomy is illustrated in Figure 3. There is a difference in the pattern of regeneration of the two groups. Untreated animals showed an initial gain in liver weight after partial hepatectomy, while the DCA-treated animals started off more slowly. At the end of 14 days both DCA-treated and untreated animals reached the same percentage of liver weight (Table 2; Fig. 3). The percentage of regeneration of the liver in each of the two groups shows the same pattern as the net liver growth, two-thirds of the original liver weight having been reached at 14 days after the operation (Table 2). Bucher and Glinos (1950) found approximately the same degree of regeneration of the liver at 14 days after partial hepatectomy in mature rats of the same approximate age as those of the present experiment. A correction for changes in body weight during the period of re-

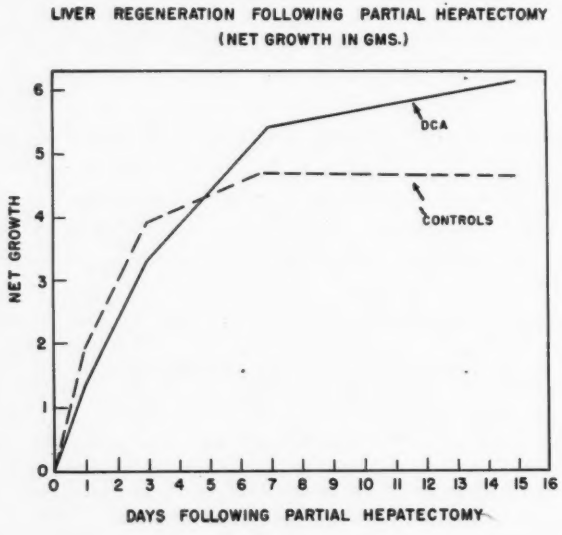

FIG. 3

TABLE 3. PER CENT WEIGHT LOSS BY THE ANIMALS IN TWO GROUPS AT STATED TIME INTERVALS AFTER PARTIAL HEPATECTOMY

Days after operation	1	3	7	14
Rats treated with desoxycorticosterone acetate	5.7	8.7	5.6	3.9
Rats not treated with desoxycorticosterone acetate	5.7	7.5	5.8	3.2

generation seemed unnecessary because there was no significant difference in the mean loss of body weight between the two groups (Table 3).

Figures for protein nitrogen, non-protein nitrogen, fat and moisture content of the liver (Table 1), show no significant differences between the DCA-treated and untreated animals. This held true for all groups at the different stages of regeneration. Protein nitrogen for the whole liver was significantly higher in DCA-treated than in untreated rats (Table 1).

Cytological evaluation of the DCA-treated and untreated groups revealed differences in the pattern of mitotic activity which reflect the changes in liver weight after partial hepatectomy in the DCA-treated and in the untreated rats (Fig. 4). The peak of mitotic activity occurred at about the third day after the operation. While the DCA-treated rats showed a prolonged phase of mitotic activity, the mitotic rate in untreated animals fell sharply after the third day (Fig. 4). The ratio of proliferation

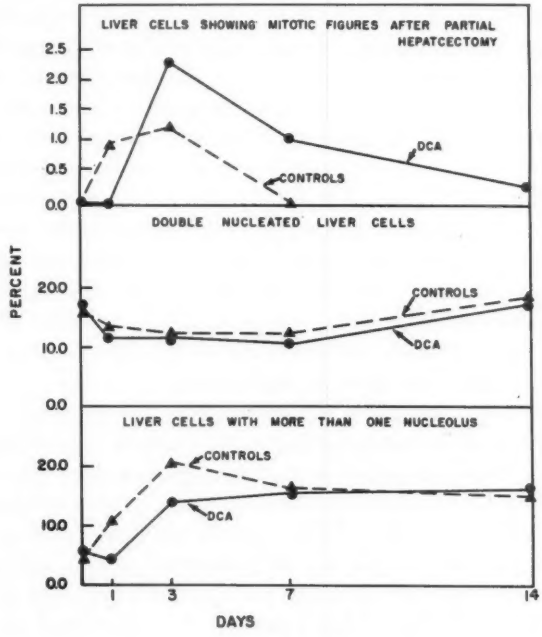

FIG. 4

of the hepatic tissue, in comparison with the number of double or multinucleated liver cells revealed an inverse relationship (Figs. 3 and 4). On the other hand, an increase in the rate of mitosis was directly related to an increase in the number of liver cells possessing more than one nucleolus (Fig. 4). Vascularity, sinusoids and number of reticuloendothelial, bile-duct cells and epithelial cells were essentially unaltered in the livers of DCA-treated rats at all stages of regeneration of the liver.

Changes in the adrenal gland: The adrenal cortex of DCA-treated rats was atrophic (Symeondis *et al.*, 1954) on day zero. This atrophy disappeared at 24 hours after the animals were partially hepatectomized. At this time, the cortical layers of these rats were enlarged and fine lipoid droplets were found in the cells, even of the zona glomerulosa. Gradually, the adrenal glands evidently reverted to their original structure, and 14 days after partial hepatectomy the adrenal cortex in each group showed more or less the same features as seen on day zero.

DISCUSSION

In view of these observations, it is of interest to note an increase in the weight of liver tissue without appreciable change in body weight under long-continued treatment of the rats with desoxycorticosterone acetate. The liver weight of the DCA-treated animals was significantly higher than that of untreated rats (Table 1), both in animals that were killed on day zero and others that were killed later. There is no increase either in fat or moisture concentration of the livers of the DCA-treated rats over those of the untreated ones, the protein nitrogen concentration (a good measure of protoplasmic material) in both groups is the same, and there is a significant increase in the quantity of protein nitrogen per liver in DCA-treated rats. Hence, it is concluded that this increase in liver weight is due to an increase in liver tissue. The cytological analysis as well as the microscopic study of the liver point to the same conclusion. The cytological analysis and the microscopic study of the liver revealed no important difference between DCA-treated and untreated rats. The ratio between the different cellular elements, the volume of the liver cells, the number of sinusoids and blood vessels, and the microscopic structure of the liver as a whole was essentially the same in both groups.

At fourteen days after partial hepatectomy, the net liver growth in the DCA-treated rats had reached a higher level than that of untreated animals. In other words, a larger liver mass developed in the DCA-treated rats after 14 days of liver regeneration. However, upon comparing the percentage of tissue restoration of the initial liver mass, no difference in the two groups was found (Table 2). DCA-treated rats as well as untreated rats regenerated about two-thirds of their initial liver mass within 14 days after partial hepatectomy. This indicates that the trend in both groups was to restore the original liver mass which was larger in the DCA-treated

animals. Accordingly, it seems that prolonged treatment wih desoxycorticosterone acetate produces in the body conditions under which a larger liver develops. High doses of DCA of 1 mg. and above per day are in this respect effective (Berman *et al.*, 1947; Friedgood *et al.*, 1950; Uyldert 1950; Fanfani and Marconi, 1951). The pattern of liver regeneration was different in the DCA-treated rats than that of the untreated animals. Regeneration of the liver in DCA-treated rats began more slowly, but exceeded that of the untreated animals after the third post-operative day (Fig. 3). The slow start of regeneration and the delay in appearance of mitotic cells (Fig. 4) in the DCA-treated rats would indicate that the desoxycorticosterone acetate depresses the initial stages of liver regeneration. Then, liver growth is promoted again after the third postoperative day. This is in agreement with the observation of Berman *et al.*, 1947 in a short-term experiment with DCA. The well-known drastic reduction of binucleated liver cells during liver regeneration (Bucher *et al.*, 1950; St. Aubin *et al.*, 1952; Wilson *et al.*, 1953) occurred in this experiment to the same extent in both groups (Fig. 4). The correlation of the rate of proliferation of liver cells with the number of liver cells containing more than one nucleolus could be considered as another useful tool for measuring rates of tissue proliferation (Figs. 1 and 2).

These findings indicate that desoxycorticosterone acetate is a promoting factor for liver growth, in contrast to cortisone which has apparently an inhibitory effect upon growth and regeneration of the liver (Einhorn *et al.*, 1954). These influences of the adrenal cortical hormones upon liver protein metabolism may contribute to our understanding the effect of adrenal cortical secretion on carcinogenesis in the liver (Symeonidis *et al.*, 1954).

Atrophy of the adrenal cortex of DCA-treated animals is known to be an inhibitory effect of DCA on ACTH secretion (Sayers *et al.*, 1947). Temporary recovery of the adrenal cortex of DCA-treated rats, to normal size and morphology, 24 hours after partial hepatectomy could be attributed to the stimulation of the pituitary gland by hepatectomy and the resulting increase of ACTH secretion.

SUMMARY

Eighteen, male Osborne-Mendel rats, 9 to 12 weeks of age, treated with subcutaneous implants of pellets of desoxycorticosterone acetate (DCA) for 28 weeks (average absorption of 1.1 mg. of the steroid, per rat per day) showed marked increase in liver mass (both on absolute liver weight and on per 100 gm. body weight basis) over that of the untreated rats. This increase in liver mass was due to an increase in liver tissue as shown by absolute increase in total liver protein per animal. The regenerating liver of partially hepatectomized rats treated with DCA developed greater liver mass than the liver of untreated rats. Treatment with DCA at first depressed the regeneration of the liver tissue, but later the mitotic ac-

tivity was increased and prolonged by this hormone. The extent of restoration of the liver was the same both in treated and untreated rats at the end of 14 days. The increase in the number of liver cells containing more than one nucleolus paralleled the rate or proliferation of the regenerating liver. Atrophic adrenal glands of the DCA-treated rats were temporarily restored to normal size and morphology, following partial hepatectomy. They reverted to their original structure 14 days after hepatectomy.

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RELATION OF CRYSTAL SIZE TO ESTROGENIC ACTIVITY OF PARAHYDROXYPROPIOPHENONE

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PARAHYDROXYPROPIOPHENONE (PHP), with a chemical formula being one half of the stilbestrol molecule, has been described as a non-estrogenic substance and as having an inhibitory action upon the secretion of gonadotrophic hormone by the anterior pituitary (Perrault, 1950). These properties have not been confirmed by all investigators. Some have not found any effect whatsoever whereas others reported that it is an estrogenic substance. The reason for these striking contradictions is hard to explain as will be seen from the discussion below. Recently, Lacassagne *et al.* (1954) have attributed the estrogenic action, when there is such, to the presence of impurities which have appeared during the process of synthesis of the substance.

Having been unable to demonstrate in rats either, an estrogenic or an inhibitory action upon the pituitary, with doses up to 50 mg. of PHP powder per day (Foglia, di Paola and Hutz, 1951) and in view of a report of Lacassagne, Chamorro and Buu-Höi (1951) in which they found action using doses as high as 120 mg., we decided to repeat the experiments with this larger dose and under new conditions.

TECHNIQUES AND PLAN

The experiments were made with white rats of about 120 gm. body weight, kept on a diet of milk, bread and supplements. (Foglia 1944). *Castrations* were performed at one month of age. The *hypophysectomized* females were used 10 days after the operation. The *vaginal smears*, taken with the help of a fine pipette loaded with physiological saline, were examined at once under the microscope without staining. *Autopsies* were performed by killing the animals with illuminating gas, then removing, weighing and keeping for histological study specimens of the following organs: pituitary, adrenals, thyroid and uterus.

All of the parahydroxypropiophenone (PHP) employed was from the same sample, which was in a powder form.[1] The powder was made up of crystals of variable size, as a general rule larger than 10,000 μ^3; the fragmentation of these crystals was so marked as to make it difficult to recognize their rhomboidal shape. From this original powder microcrystals were prepared by dissolving it in one volume of ethanol and one volume of propyleneglycol at 90°–95° C and then crystallizing it from the cold solution, by adding

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[1] Kindly supplied for the purpose by the Laroche-Navarron Laboratories in Paris.

8 volumes of a 0.01% of a detergent[2] in twice-distilled water; microcrystals of any desired size may be obtained by changing the speed at which the detergent is added to the solution of PHP. Each cc. of this mixture contained 50 mg. of microcrystals. In order to inject small quantities, of microcrystals, this mixture was diluted with a liquid made up of 10% ethanol, 10% propylene glycol and 80% detergent, i.e., the same as the supernatant liquid. The usual volume of the microcrystals was 2,000 μ^3 except in special cases later to be mentioned. The original powder and the microcrystals displayed identical melting points and absorption curves in ultraviolet light.

The plan was to study the actions of the original powder and of the microcrystals of PHP on groups of 10 rats, accompanied by their corresponding untreated controls.

The *original powder* in daily doses of 120 mg. was administered orally to: a) castrated males; b) normal males and females; and c) spayed normal females.

The *microcrystals* were given orally or by injection into spayed female rats varying the size of the microcrystals, the dose and the time of administration and studying the variations in a) the vaginal smear and b) the body-weight and the weights of various organs.

RESULTS

A) *PHP original powder.*—After giving orally 120 mg. daily for 15 days to castrated male rats, there was no change in the amount of *growth, thyrotrophic, corticotrophic* and *gonadotrophic* hormones in the hypophysis. This was shown by injecting one of these pituitaries daily for 10 days into hypophysectomized female rats[4] and recording the body-weight and at autopsy the weight of various organs. The controls received pituitaries from untreated animals.

After giving the same dose for 2 months to normal rats of both sexes, there was no significant effect of PHP on the body-weight, and on the weights of the endocrine glands, the secondary sexual organs, the liver and the kidneys.

Castrated female rats which were observed for 2 months, remained in dioestrus even though they received 120 mg. of the drug daily. In normal females with the same dose and observation period, the number of estrus cycles was slightly, but not statistically significantly (treated, 7 ± 1 cycles; untreated, 10 ± 0.7), diminished.

B) *PHP in microcrystals.*—Vaginal estrous was not produced in castrated rats which received *orally* 150 mg. of the original powder daily for 2 days. On the other hand, administration of microcrystals having an average volume of 2,000 μ^3 and prepared from this powder sample to the same animals and by the same route produced estrus in all of them in daily doses of 150, 50 and 10 mg.; with a dose of 5 mg. estrus was observed in 50% (Table 1).

[2] Benzalkonium chloride.

[3] The size of the crystals mentioned refers to the average, as among the total there are larger and smaller ones. The PHP forms rhomboidal crystals and under the microscope two of their sides can be measured while the third side was taken arbitrarily to be equal to the shorter; nevertheless the expression of the size in volume, even if it is an approximation, furnishes a more real idea than the indication of the surface area.

[4] A total of 10 fresh pituitaries to each hypophysectomized rat.

TABLE 1. EFFECT OF ORAL ADMINISTRATION OF PARAHYDROXYPROPIOPHENONE IN POWDER FORM AND IN MICROCRYSTALS (BOTH OBTAINED FROM THE SAME SAMPLE) ON ESTRUS

Parahydroxy-propiophenone:	Daily dose for 2 days	Incidence of vaginal estrus in 10 spayed rats. Days after cessation of treatment				
	mg.	1	2	3	4	5
Original powder	150	0	0	0	0	0
Microcrystals (2,000 μ^3)	150	10	10	8	6	0
	50	10	10	7	5	0
	10	10	5	4	1	0
	5	3	6	1	0	0
	5	3	4	1	0	0

When the original powder was suspended in 0.9% Na Cl or in corn-oil, it did not produce estrus in the same group of animals in daily doses (subcutaneous) of 150 and 50 mg. respectively. Suspended in sesame oil, it was active in doses of 25 mg. in all the rats (Table 2). The microcrystals (2,000 μ^3), obtained from this original powder and injected, produced estrus in 100% of the cases, with daily doses of 150, 50, 10, 1, 0.5 and 0.25 mg. and in 50% with 0.05 mg. Both in these experiments *by injection* and in the previous ones *by mouth*, the estrogenic effect lasted but a few days even with the highest doses.

The clear supernatant obtained after letting the microcrystals settle for several hours has estrogenic activity. The mixture of only ethanol, propyleneglycol and detergent, added together in the same way and the same proportions as done when preparing microcrystals, did not provoke estrus in the castrated rat. The microcrystals retain their estrogenic activity, even when prepared and suspended in only two of the three liquids which are used in the crystallization procedure (Table 3).

Microcrystals of different size, though of the same shape, obtained from

TABLE 2. EFFECT OF SUBCUTANEOUS INJECTION OF PARAHYDROXYPROPIOPHENONE IN POWDER FORM AND IN MICROCRYSTALS (BOTH OBTAINED FROM THE SAME SAMPLE) ON ESTRUS

Parahydroxypropiophenone:	Daily dose for 2 days	Incidence of vaginal estrus in 10 spayed rats. Days after treatment:							
	mg.	1	2	3	4	5	6	7	8
Original powder in 0.9% NaCl	150	0	0	0	0	0			
Original powder in corn oil	50	0	0	0	0	0			
Original powder in sesame oil	25	3	10	2	0	0			
Microcrystals (2,000 μ^3)	150	10	10	10	10	8	7	3	0
	50	10	10	10	6	4	0	0	0
	10	8	9	10	10	2	0	0	0
	1	6	10	10	5	2	0	0	0
	0.5	5	10	10	6	0	0	0	0
	0.25	4	6	10	7	1	0	0	0
	0.05	2	4	5	2	0	0	0	0

TABLE 3. EFFECT OF THE FLUID OF SUSPENSION OF MICROCRYSTALS

Microcrystals of Parahydroxypropiophenone (2,000 μ^3)	Daily dose for 2 days	Incidence of vaginal estrus in 10 spayed rats Days after treatment				
	mg.	1	2	3	4	5
Liquid which usually suspends them: (1)						
a) supernatant	0.5 cm³	4	10	6	0	0
b) control (not in contact with the microcrystals)	0.5 cm³	0	0	0	0	0
Prepared and suspended in (2)	1	10	10	5	0	0
Prepared and suspended in (3)	1	9	10	4	0	0
Prepared and suspended in (4)	1	6	10	6	2	0

[1] 10% ethanol+10% propyleneglycol+80% sol. 0.01% benzalkonium chloride.
[2] 10% ethanol+90% sol. 0.01% benzalkonium chloride.
[3] 10% propyleneglycol+90% sol. 0.01% benzalkonium chloride.
[4] 10% ethanol+10% propyleneglycol+80% sol. 0.1% NaCl.

the same inactive sample of PHP original powder show that those of 400 and 2,000 μ^3 produce estrus in doses as small as 0.05 mg. per spayed rat whereas those of 3,000, 4,500 and 8,000 μ^3 are inactive even in doses of 5 mg. per rat and per day (Table 4). Microcrystals of diethylstilbestrol of 4,600 μ^3 in doses of 0.5 mg. are still active 15 days after injection.

On transforming microcrystals of 2,000 μ^3 into smaller crystals of the same shape 10 μ^3, the estrogenic action upon injection into spayed rats persists. If the latter are transformed to larger crystals, with overage volume of 10,000 μ^3, the action disappears, on changing them back to the initial size of 2,000 μ^3 it is restored. The injected dose was 2.5 mg. per day and the shape of the microcrystals was the same (Table 5).

TABLE 4. EFFECT OF SIZE OF MICROCRYSTALS OF PARAHYDROXYPROPIOPHENONE

(All samples were prepared directly from the same original powder)

Size of Microcrystals injected:	Daily dose for 2 days	Incidence of vaginal estrus in 10 spayed rats Days after treatment:					
	mg.	1	2	3	4	5	6
Parahydroxypropiophenone (400 μ^3)	1	2	10	10	6	0	0
	0.5	2	10	4	2	0	0
	0.25	1	10	2	0	0	0
	0.10	0	3	5	0	0	0
	0.05	0	2	2	0	0	0
Parahydroxypropiophenone (3,000, 4,500 and 8,000 μ^3)[1]	5	0	0	0	0	0	0
	1	0	0	0	0	0	0
	0.5	0	0	0	0	0	0
Diethylstilbestrol	1	8	10	10	10	10	10 (persisted for 16 days).
(4,600 μ^3)	0.5	6	10	10	10	10	10 (persisted for 15 days).

[1] Each size of microcrystals was tested at each of the 3 different dose levels.

TABLE 5. EFFECT OF SIZE OF MICROCRYSTALS OF PARAHYDROXYPROPIOPHENONE
(*Each sample was prepared by recrystalization of the preceding sample in the table*)

Size of microcrystals of parahydroxypropiophenone.	Daily dose for 2 days	Incidence of vaginal estrus in 10 spayed rats. Days after treatment					
	mg.	1	2	3	4	5	6
10,000 μ^3	2.5	0	0	0	0	0	0
↓							
2,000 μ^3	2.5	5	10	10	6	2	0
↓							
10 μ^3	2.5	10	10	10	7	3	0
↓							
10,000 μ^3	2.5	0	0	0	0	0	0
↓							
2,000 μ^3	2.5	6	10	9	5	1	0

TABLE 6. EFFECT OF PARAHYDROXYPROPIOPHENONE ON BODY AND ORGAN WEIGHTS[1]

	No. of rats	Body weight, gm.			Hypophysis mg.	Thyroid mg.	Adrenals mg.	Uterus mg.
		Initial	Final	Gain				
Parahydroxypropiophenone	10	150	173	23	15.9±0.8[2]	28.4±2.3	51.2±4.5	327±64
Controls	10	150	197	47	10.3±0.8	24.6±1.6	38.4±1.7	81± 5.3

[1] Parahydroxypropiophenone in microcrystal size 2,000 μ^3, injected 5 mg./day, for 14 days. Controls injected with solvent only, 1 cc. a day for 14 days.
[2] Mean ± standard error.

The microcrystals of active size (2,000 μ^3) injected in doses of 5 mg. daily, for 2 weeks, into spayed female rats increased the weight of the hypophysis, the adrenals and specially the uterus (Table 6).

DISCUSSION

The sample of PHP *original powder* utilized in these experiments proved inactive in rats in spite of the high dose (120 mg. daily) and the protracted time (2 months) in which it was administered, thus confirming researches of ours carried with lower doses (Foglia, di Paola, Hutz, 1951). Our results are not in agreement with those of Lacassagne *et al.*, (1951) in spite of the fact we used the same daily dose and for twice the time. Employing the *subcutaneous* route, 25 mg. of the original powder per rat in sesame oil was active, thus revealing the importance of the medium of suspension (Table 1).

The administration of *microcrystals*, of 2,000 μ^3 obtained from the previous inactive sample of original powder to spayed female rats was most effective. *Orally*, and by *subcutaneous injection* it caused vaginal estrus of short duration in 100% of the animals, body growth was moderated and there was an increase in the weights of the pituitary, adrenals and especially the uterus (Tables 1, 2 and 6). All these are characteristic phenomena that estrogens are known to produce in spayed animals: hypertrophy of the uterus and vaginal estrus by direct action upon these organs, and, upon the hypophysis, increase in its weight, decrease in growth hor-

mone and increase in corticotrophin content.

The production of vaginal estrus by the microcrystals is found to be connected with their size and not with their shape which always remains unchanged. In fact, those of 10, 400 and 2,000 μ^3 in daily doses of 0.25 mg. per rat are active in 100% of the cases, and those of 3,000, 4,500, 8,000 and 10,000 μ^3 are inactive even in doses of 5 mg. (Tables 4 and 5). It is important to point out that this result was observed both when preparing the crystals straight from the original powder and when retransforming them from one size to another on repeated occasions (Table 5). These experiments are not very favourable to the hypothesis of the production of some estrogenic substance during the manipulations of their preparation.

The mixture of liquids that maintains the microcrystals in suspension, if prepared without contact with them, does not produce vaginal estrus. The estrogenic action of the microcrystals does not appear to be modified even when the mixture is prepared without one of its three components. Once in contact with the microcrystals and separated by decantation it becomes estrogenic (Table 3).

All the foregoing results might be explained by supposing that the estrogenic action of the PHP depended upon its absorption speed. This line of thought would be supported by the following considerations: a) the inactive original powder and the active microcrystals, obtained from them, possess the same physical characteristics as regards their melting point and absorption curve to ultraviolet light, which suggests their chemical similarity[5]; b) the original powder acquires estrogenic activity with a suitable solvent (sesame oil) or through transformation into microcrystals of the proper size; c) the microcrystals of 2,000 μ^3 or less fail to produce vaginal estrus when transformed into others of a larger size and when restored to their first size they recover this power.

SUMMARY AND CONCLUSIONS

The administration of parahydroxypropiophenone original powder to rats in a high dose sustained for a long time, orally or by subcutaneous injection (in saline or corn-oil) did not provoke estrus nor inhibit the pituitary.

The same powder acquired estrogenic activity when injected in sesame oil or transformed into microcrystals of 2,000 μ^3 or less.

The microcrystals were active orally and parenterally and produced all the typical phenomena of estrogenic substances.

Active microcrystals of 2,000 μ^3 or less when transformed into larger ones (3,000 to 10,000 μ^3) of the same shape become inactive. If these large microcrystals are retransformed into small ones they recover their estrogenic activity.

[5] More extensive analysis are required in order to establish this point.

All these results indicate that the activity of PHP is dependent on the size of its crystals.

The supernatant liquid obtained by decantation from the microcrystals also has estrogenic activity.

In conclusion, PHP appears to be a weak estrogenic substance, the action of which depends upon the rate of absorption by the animal.

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THE BIOLOGICAL ACTIVITY OF O-METHYL THYROXINE[1]

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AN INVESTIGATION of the O-methyl ether of thyroxine was prompted by conflicting evidence in the literature concerning the biological activity of this compound and by the possibility that *in vivo* methylation of thyroxine is concerned either with the biosynthesis of the cellularly-active form of the hormone or with the detoxication of thyroxine.

O-Methyl-DL-thyroxine was first synthesized by Myers (1932) by reaction of N-acetyl thyroxine with dimethyl sulfate. The methyl ether (m. p. 210–213°) obtained on deacetylation was reported to be less active than thyroxine in stimulating amphibian metamorphosis, and inactive when administered intravenously or subcutaneously to human subjects.

In contrast, Loeser, Ruland and Trikojus (1938), who prepared O-methyl-DL-thyroxine (m. p. 226–228°) by direct methylation of thyroxine or thyroxine methyl ester with diazomethane, reported the compound to be *more effective than thyroxine* in depressing body weight, increasing basal metabolic rate, and in decreasing liver glycogen of guinea pigs. However, somewhat later, Clayton and Hems (1950) reported thyroxine methyl ether to be only half as active as thyroxine in stimulating oxygen consumption of mice, and Lerman, Harington and Means (1952) found it to be only 5 to 6% as effective as L-thyroxine in stimulating basal metabolic rate of myxedematous patients. Loeser *et al.* administered their preparations orally. Therefore the observed, comparatively greater effectiveness of the ether may have resulted from relatively poor absorption of thyroxine by the oral route.

These observations were of academic interest only until Maclagen and Wilkinson (1951) found that patients fed *n*-butyl-3,5-diiodo-4-hydroxybenzoate excreted in their urine the methyl ether of the corresponding free acid. This was apparently the first recorded example of O-methylation in the mammalian body. Since thyroxine possesses the same *ortho*-diiodophenolic group, it seemed possible that its *in vivo* methylation might be involved in the normal metabolism of the hormone.

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EXPERIMENTAL

O-Methyl-DL-thyroxine was synthesized from thyroxine[2] by the method of Loeser, Ruland and Trikojus (1938). After 3 recrystallizations the compound melted at 224°; Clayton and Hems (1950) reported the same melting point.

Complete absence of thyroxine in our preparation was indicated by a negative Kendall test and by the absence, in alkaline solution, of detectable absorption at 325 mμ where the characteristic phenolic absorption of thyroxine occurs. The compound is even more difficultly soluble than thyroxine. For biological testing it was dissolved in propylene glycol and a little 0.1 N KOH. It was then brought to the desired volume and a final concentration of 50% water–50% propylene glycol. All compounds were administered to rats subcutaneously.

RESULTS

Effect on tadpole metamorphosis.—The methyl ether of thyroxine was assayed by the procedure of Bruice, Winzler and Kharasch (1954) except that *Rana clamitans* tadpoles rather than *Rana catesbiana* were used, and

TABLE 1. EFFECT OF O-METHYL THYROXINE ON METABOLIC RATE OF RATS

Experiment	Daily Dose of Thyroxine	Mean ±S.D. of Oxygen Consumption	Daily Dose of O-Methyl-DL-Thyroxine	Mean ±S.D. of Oxygen Consumption
	mg./kg.	L./Sq.M./Hr.	mg./kg.	L./Sq.M./Hr.
1	None	7.4 ±1.0		
Sept., 1953	.075 (DL)	8.5 ±0.8	.075	7.7 ±0.6
6 rats/lot	.20 (DL)	9.3 ±0.6	.20	8.1 ±0.6
2	None	6.8 ±0.5		
Oct., 1953	.075 (DL)	7.9 ±0.8	.075	7.3 ±0.8
6 rats/lot			.20	8.2 ±1.1
3	None	6.8 ±0.6		
Sept., 1954	.10 (DL)	8.3 ±0.9	.30	8.3 ±0.4
5 rats/lot	.15 (DL)	8.7 ±1.7	.45	9.0 ±1.9
	.30 (DL)	9.6 ±0.65	.90	8.9 ±1.3
4	None	8.0 ±0.88		
March, 1955	0.25 (L)	9.7 ±0.93	.5	9.1 ±0.50
5 rats/lot	1.0 (L)	10.5 ±1.51	2.0	9.9 ±0.72
	2.0 (L)	10.3 ±0.95	4.0	10.0 ±0.68

they were exposed to the hormone for only 24 hours. At concentrations of one and two parts per million the methyl ether caused slightly greater tail shrinkage than equivalent concentrations of L-thyroxine. Since L-thyroxine is nearly twice as active as the DL mixture in this assay, O-methyl-DL-thyroxine is approximately twice as active as free DL-thyroxine. In two other assays with *Rana pipiens* tadpoles the methyl ether was slightly more effective than equivalent concentrations of DL-thyroxine in stimulating the appearance of the forelimbs.

Effect on oxygen consumption of rats.—In these experiments thyroxine or

[2] DL-thyroxine was generously supplied by Dr. E. Severinghaus and Dr. M. J. Schiffrin of Hoffmann-LaRoche, Inc.

the methyl ether were injected at the indicated dose level for 3 consecutive days. The rats were deprived of food after the third injection and oxygen consumption was measured on the fourth day as described by Holtkamp *et al.* (1955).[3] Four separate experiments, in each of which two or three levels of the methyl ether were administered, indicated that this compound possesses about $\frac{1}{3}$ the activity of DL-thyroxine (Table 1). This relationship is more clearly brought out in Figure 1. At the two lower levels of assay, the methyl ether gave the same response as $\frac{1}{3}$ as great a dose of DL-thyroxine. However, 0.9 mg. of the methyl ether per kg. daily was no more effective than half that amount.

Goiter-prevention assay.—The methyl ether of thyroxine was tested for

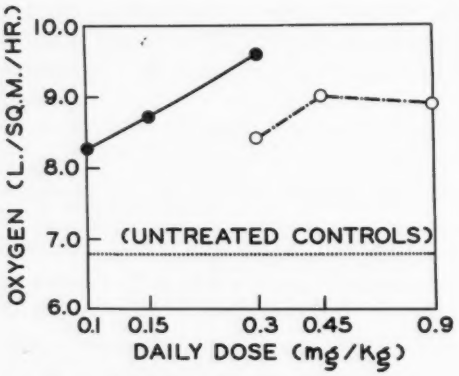

FIG. 1. Effect of O-Methyl-DL-thyroxine on Oxygen Consumption of Rats. Each point represents the mean oxygen consumption of 5 rats. Thyroxine treatment = ●; O-methyl-DL-thyroxine = 0. Untreated control rats consumed 6.8 liters per square meter body surface per hour.

its ability to inhibit pituitary secretion of thyroid stimulating hormone in rats fed a diet containing 0.3% thiouracil. Six male rats of approximately 100 gm. body weight were used in each lot. They were sacrificed after having received 10 daily injections of the methyl ether or the standard reference substances, thyroxine and tri-iodothyronine. In three preliminary assays the methyl ether, at levels up to 20γ daily, showed no detectable activity. Coming after the two previously described assays, this result was entirely unexpected. In a final assay it showed some activity when 40γ were administered daily although there was great variability among the rats in their response (Table 2).

To determine whether the ether would inhibit the response to thyroxine, the rats of one lot were given 6γ thyroxine and 20γ of the methyl ether

[3] These measurements were made in the laboratories of Smith, Kline and French, and I am indebted to Dr. D. E. Holtkamp and Dr. A. E. Heming for them.

TABLE 2. EFFECT OF O-METHYL-DL-THYROXINE ON THYROID WEIGHTS OF THIOURACIL-FED RATS

Treatment	Thyroid wt./100 gm. body wt.
	mg.
Untreated Control	7.5±1.0
Thiouracil alone	31.2±3.7
Thiouracil+2γ DL-Thyroxine	22.9±5.5
Thiouracil+4γ DL-Thyroxine	10.2±3.5
Thiouracil+6γ DL-Thyroxine	7.8± .7
Thiouracil+ 2γ O-Methyl-DL-Thyroxine	29 ±3.7
Thiouracil+ 8γ O-Methyl-DL-Thyroxine	30 ±5.3
Thiouracil+20γ O-Methyl-DL-Thyroxine	31 ±6.4
Thiouracil+40γ O-Methyl-DL-Thyroxine	20 ±8.6
Thiouracil+20γ O-Methyl-DL-Thyroxine and 6γ DL-thyroxine	8.4±3.2

daily. The mean thyroid weigths of these rats was not different from that of the rats receiving 6γ thyroxine daily.

If one were to accept the value of the single lot which received 40γ of the methyl ether, this compound would be recorded as 7 per cent as active as DL-thyroxine. In view of the consistently negative results in repeated assays at lower levels, it seems doubtful whether the ether possesses even this low an order of activity in the Dempsey-Astwood (1943) assay.

DISCUSSION

The finding that O-methyl thyroxine is fairly effective in stimulating oxygen consumption of rats and mice while ineffective, or nearly so, in the goiter prevention assay, marks it as different from most of the derivatives and analogues of thyroxine which have been studied. An important difference in the conditions of the two assays is the presence of thiouracil in the diet of the rats in the goiter-prevention studies. If the methyl ether *per se* were inactive but were converted to free thyroxine *in vivo* by a process which was inhibited by thiouracil, the methyl ether would appear to be active in the oxygen consumption assay and would be inactive in the Dempsey-Astwood assay. To test this possibility, an experiment has been conducted with rats fed 0.3 per cent thiouracil (Table 3). Thiouracil feed-

TABLE 3. EFFECT OF O-METHYL-DL-THYROXINE ON METABOLIC RATE OF THIOURACIL-FED RATS

Treatment	Oxygen Consumption Mean ±S.D.
	L/Sq.M./Hr.
Control	7.53±0.95
Thiouracil (0.3 per cent in diet)	6.84±0.28
Thiouracil+O-Methyl thyroxine (0.45 mg./Kg. daily)	8.37±1.07
Thiouracil+O-Methyl thyroxine (0.3 mg./Kg. daily)	7.18±0.40
Thiouracil+Na L-Thyroxine (0.075 mg./Kg. daily)	8.41±1.24
Thiouracil+Na L-Thyroxine (0.05 mg./Kg. daily)	7.57±0.38

Six rats per lot. Thyroxine and the methyl ether were administered on the fourth, fifth, and sixth days of thiouracil feeding and metabolism was measured on the seventh day.

ing did not prevent the effect of O-methyl-DL-thyroxine on metabolic rate and therefore it appears not to be responsible for the lack of effect of the methyl ether on thyroid gland weight.

Since the methyl ether of thyroxine stimulates metabolism but fails to place a regulatory "brake" on the pituitary, it might merit further study as a possible factor in thyrotoxicosis. Recently Maclagen and Wilkinson (1954) have reported that patients suffering from thyrotoxicosis excreted a greater fraction of test doses of *n*-butyl-3,5-diiodo-4-hydroxybenzoate in the form of the methyl ether than did a normal subject or a myxedematous patient. It therefore seems worth while to determine whether thyroxine is being methylated more extensively in such patients and whether it may be stimulating metabolic rate without affecting the pituitary's production of TSH.

It is possible that the methyl ether of thyroxine is cleaved intracellularly before it becomes active. If the free thyroxine thus formed did not pass into the general circulation, and if the pituitary were unable to bring about the cleavage, the lack of effect in goiter prevention could readily be explained. If the methyl ether of thyroxine is active without being cleaved intracellularly to form the free phenolic compound, it will be necessary to revise current theory concerning the role of the phenolic group in the function of the hormone (Frieden and Winzler, 1948; Niemann, 1950).

SUMMARY

O-Methyl-DL-thyroxine was approximately twice as active as DL-thyroxine in causing tail shrinkage in *Rana clamitans* tadpoles. It was one-third as effective as DL-thyroxine in stimulating oxygen consumption of rats but was only slightly active in preventing thyroid enlargement of thiouracil-fed rats. Thiouracil feeding did not prevent the effect of O-methyl thyroxine on basal metabolic rate of rats.

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THE DISTRIBUTION OF ZINC-65 IN THE RAT PANCREAS[1]

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ALTHOUGH the pancreas appears to be involved directly in zinc metabolism, little is known concerning the role of the metal in the various tissues of this organ. Sheline and his collaborators (1943) have found that injected radioactive zinc accumulates in relatively high concentrations in the pancreas. Excretion of a major part of the injected metal in the pancreatic juice (Montgomery, Sheline, and Chaikoff, 1943) and extraction of a zinc-containing enzyme from the pancreas (Vallee and Neurath, 1954) imply activity of the acinar tissue. On the other hand, numerous reports suggest that zinc might also be involved in islet cell function. It is well known, for example, that zinc facilitates the crystallization of insulin (Scott, 1934) and tends to prolong its biological action when present in suitable concentration (Hallas-Møller, *et al.*, 1952). Furthermore, diabetogens such as alloxan and dithizone cause a decrease in islet zinc content as estimated by histological methods (Stampfel, 1951). Alloxan is also reported to reduce the uptake of radiozinc by the pancreas (Lowry, 1954), but the locus and mode of action are unknown. Since the pancreas is not a homogeneous organ but has two or more distinct functions, studies of zinc uptake in the whole gland give little information about its distribution among the various component tissues. In the present study, differences in the distribution of zinc in the two major tissues of the pancreas, acini and islets, have been demonstrated by an autoradiographic technique.

EXPERIMENTAL

Male and female rats, Wistar strain, weighing between 150 and 250 gm. were anesthetized with Pentobarbital Sodium 50 mg. per Kg. and radioactive zinc-65 chloride was injected into an exposed femoral vein. The solution for injection contained 100 microcuries of zinc-65 per ml. of solution. The amount administered was in most cases between 100 and 150 μc. per 100 gm. of body weight. However, in one instance it was as low as 75 μc. and in two instances, as high as 200 μc. per 100 gm. body weight. Two separate solutions were used, one having a specific activity of 370 μc./mg. zinc and the second, 520

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μc./mg. Control animals were injected with solutions containing non-radioactive zinc chloride. The rats were housed in metabolism cages (Brunner, 1951), permitting the separation of urine and feces.

At intervals ranging from 4 to 92 hours after injection, animals were anesthetized with chloroform and exsanguinated. The pancreas was quickly removed, cut into small pieces, and immediately frozen in liquid nitrogen. The tissue was fixed by the freeze-dehydration method of Holt, Cowing, and Warren (1949) to prevent leaching or translocation of isotope. It was blocked in paraffin.

In order to prepare the autoradiographic slides, sections were cut 10μ thick and the wrinkles removed by carefully warming and pressing them flat (Gallimore, Bauer, and Boyd, 1954). The tissue sections were transferred to the darkroom and pressed onto NTB-2 autoradiographic plates (Eastman Kodak).[3] The film was placed in a light-tight box which contained calcium chloride to maintain low humidity and stored in a refrigerator for the duration of the exposure. The films with the adherent sections were then immersed in 2 changes of xylol to remove the paraffin and brought through acetone and alcohol to water. They were developed in D-19 (Eastman Kodak) for 10 minutes at 20° C., rinsed, fixed, washed, and stained with acid hematoxylin and eosin.

The relative concentration of radioactive zinc in the islet and acinar tissues of the pancreas was determined by counting grains in the photographic emulsion immediately under any chosen area of tissue. A reticle which was divided into 100 small squares was used to outline the area counted. The oil immersion objective was brought over islet tissue, and all the grains in the film under the four small squares at each corner and at the center of the reticle were counted while slowly racking the point of focus down through the film. Each count was repeated three times as a check on its accuracy. Thus, five areas, representing 100 square microns each, were evaluated for each islet and the average of the counts from the five small areas at each position was calculated. An identical procedure was followed for a portion of the acinar tissue close to the counted islet. Background counts were made in a similar manner, but of an area of film not covered by tissue.

The individual counts from slides of the same animal and same expsosure time were averaged after the background count had been subtracted. These averages were used in the calculations and interpretations. The mean islet and acinar grain counts were analyzed by the "t" test to determine the significance of the difference between the means. If P was found to be less than 0.01, the means were considered to be significantly different. The ratio of islet to acinar grain counts was calculated for each series of slides in order to establish a convenient basis for comparison of one animal with another. This procedure was necessary to prevent errors due to differences in dose, time of decay, and exposure times. These ratios indicate the islet cell zinc-65 concentration relative to the acinar concentration. Since acinar tissue comprises the major fraction of the pancreas, the radioactivity of the whole pancreas (Table 3) is a good indication of the approximate acinar zinc-65 concentration.

Various organs and excreta were analyzed for radioactive zinc-65 by dissolving them in concentrated nitric acid. A 1.0 ml. aliquot was placed in a plastic sample cup[4] and

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[3] The 1″×3″ NTB-2 plates used had an emulsion thickness of 10 microns and were covered with an anti-abrading coat 1 micron thick.

[4] The sample cup is made from the plastic stopper of a 7 dram glass vial (Kimble Opticlear Vial K-7). When the circular lid of the stopper is removed, a compartment with a 3 ml. volume is found. A sample is put into this compartment and the lid replaced, making a water and vapor-tight container which is suitable for counting liquid samples.

the radioactivity determined with a gamma Geiger tube (Tracerlab TGC-8). The activity of the samples was compared to that of an aliquot of the solution used for injection. The counting time in all cases was sufficient to insure a standard deviation of less than 1%.

RESULTS

Since chemical fogging of film by animal tissue has been reported (Boyd and Board, 1949), a control group of animals injected with non-radioactive zinc chloride solution was studied. In autoradiograms prepared from this

TABLE 1. THE EFFECT OF NON-RADIOACTIVE TISSUE ON NBT-2 AUTORADIOGRAPHIC FILM

Rat No.	No. Areas Counted	Average Grain Count		
		Islet	Acinar	Background
1	6	3 ± 1*	3 ± 1	2 ± 1
2	6	2 ± 1	2 ± 1	2 ± 2

* Average grain count in film under tissue ± standard deviation.

group both acinar and islet grain counts were of the same order as the background count (see Table 1). Thus, it is clear that the effect noted on films exposed to tissue from rats injected with zinc-65 is due to the particles emitted during radioactive decay rather than to chemical fogging.

Studies were carried out on rats sacrificed at 4, 7, 24, 48, 68, and 92 hours after injection, 2 rats being included at each interval. The data from the autoradiographs are summarized in Table 2.

The autoradiographic studies showed clearly that retention of radiozinc in islet tissue was different than in acinar tissue. Although the radioisotope quickly entered both islet and acinar cells, the islet concentration was less than that of the acinar tissue in the initial period after injection. This difference is reflected in the ratios of islet to acinar grain counts which were found to be 0.69 and 0.72 at 4 and 7 hour intervals, respectively.

A definite change in isotope distribution was found in rats sacrificed 48 hours or more after injection. Acinar tissue now contained only small amounts of radioactive zinc while the retention of zinc-65 in islet tissue was clearly visible using either low or high power objectives. The higher islet content persisted up to 92 hours after injection which was the longest time interval studied. The relative difference in zinc concentrations during these intervals is indicated by the islet to acinar grain ratios which were 3.0 at 48 hours, 3.4 at 68 hours, and 3.9 at 92 hours.

The general distribution of zinc-65 in blood, heart, spleen, skeletal muscle, liver, kidney, pancreas, intestine, urine, and feces is recorded in Table 3 and Figure 1. Injected zinc-65 quickly left the blood stream and appeared in high concentrations in the pancreas, intestines, liver, and kidney within 7 hours. A large part of the metal in these tissues was

TABLE 2. GRAIN COUNTS AND GRAIN COUNT RATIOS FROM AUTORADIOGRAMS OF THE RAT PANCREAS

Rat No.	Interval Injection to Sacrifice (hours)	Autograph Exposure time (days)	Average Grain Counts†		Ratio Islet Count / Acinar Count
			Under Islet Tissue	Under Acinar Tissue	
8	4	7 (16)[1]	7 ± 3	11 ± 3	0.64*
20	4	4 (35)	13 ± 5	18 ± 6	0.72*
Average					0.69
9	7	12 (7)	60 ± 16	81 ± 24	0.74*
19	7	5 (14)	26 ± 9	39 ± 11	0.67*
		6 (18)	22 ± 5	29 ± 7	0.76**
		7 (15)	32 ± 6	45 ± 6	0.71*
Average					0.72
10	24	9 (5)	76 ± 9	71 ± 12	1.1
11	24	10 (6)	61 ± 11	50 ± 9	1.2
Average					1.2
12	48	8 (15)	23 ± 5	7 ± 3	3.3*
		9 (8)	18 ± 4	5 ± 3	3.6*
		10 (5)	25 ± 3	7 ± 3	3.6*
14	48	8 (5)	24 ± 4	9 ± 3	2.7*
		10 (16)	32 ± 7	14 ± 4	2.3*
Average					3.0
16	68	8 (6)	40 ± 8	11 ± 2	3.6*
		9 (18)	36 ± 7	10 ± 3	3.6*
		10 (32)	36 ± 7	12 ± 3	3.0*
17	68	9 (63)	32 ± 7	9 ± 3	3.6*
Average					3.4
18	92	10 (43)	44 ± 11	12 ± 5	3.7*
24	92	10 (4)	27 ± 9	8 ± 4	3.4*
		14 (18)	50 ± 11	12 ± 4	4.2*
		17 (32)	56 ± 6	14 ± 3	4.0*
Average					3.9

† Average grain count in film under tissues ± standard deviation.
* The mean islet and acinar grain counts are significantly different at the 1% level.
[1] Figures in parentheses are the number of areas counted.

quickly lost and only small changes in concentration were found after 48 hours. The blood and skeletal muscle contained the lowest concentrations of the organs studied and showed but little change after the initial accumulation.

By making certain assumptions, the concentration of zinc-65 in islet tissue may be estimated. In order to simplify the calculations it is necessary to assume that the pancreas consists only of islet and acinar tissue and that the islet organ volume is a constant fraction of the total pancreatic volume in all the rats studied. As a matter of fact, the islet volume has been found to vary considerably from animal to animal, but varia-

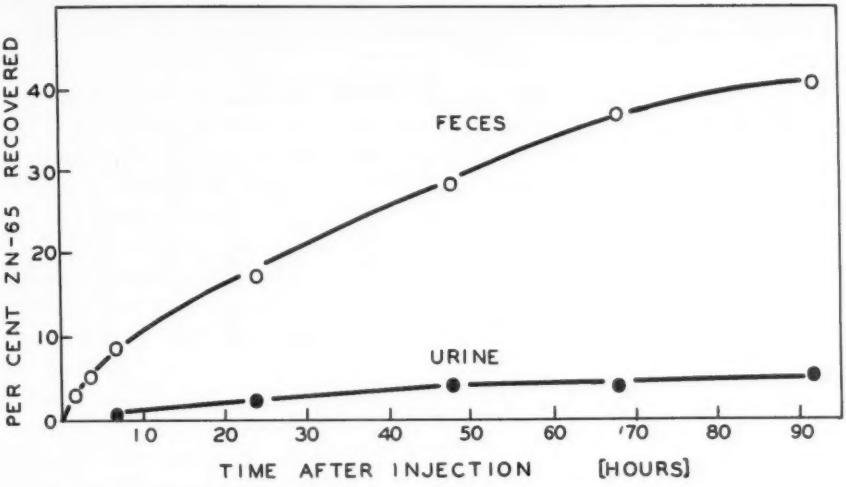

FIG. 1. Urinary and fecal excretion of zinc-65 by rats after intravenous injection of zinc-65 chloride.

tions in islet volume would have little effect upon the calculated islet zinc-65 concentration. If x is the fractional islet volume of the pancreas and $(1-x)$ the fractional acinar volume, then

$$xA_i + (1 - x)A_a = A_p$$

where A_i, A_a, and A_p are the activity per gm. tissue of islet, acinar, and whole pancreas, respectively. From autoradiographic data the ratio:

$$\frac{A_i}{A_a} = R$$

was calculated, R being the islet to acinar grain count ratio. Hence:

$$A_a = \frac{A_i}{R}$$

may be substituted into the initial equation to eliminate the term A_a:

$$xA_i + \frac{(1 - x)A_i}{R} = A_p.$$

Solving for A_i:

$$A_i = \frac{A_pR}{xR + (1 - x)}$$

The value of x (1 per cent) used in calculating the islet isotope concentration lies within values determined for the rat by Haist and Pugh (1948).

TABLE 3. CONCENTRATION OF RADIOACTIVE ZINC IN VARIOUS TISSUES AFTER INJECTION OF ZN-65

Time from Injection to Sacrifice	Zinc-65 Concentration Per cent of Injected Dose Per Gm. Tissue									
	Pancreas			Liver	Intestines	Kidney	Spleen	Skeletal Muscle	Heart	Blood
	Whole[1] gland	Acinar[2]	Islet[3]							
2	1.85	—	—	1.23	1.14	1.61	.73	.11	.75	1.07
4	3.11	3.11	2.16	1.77	.79	1.39	.62	.07	.48	.59
7	(3.00)	3.00	2.17	1.87	2.60	1.41	.88	.07	.38	.41
24	1.41	1.41	1.69	1.59	.35	.63	.41	.08	.27	.09
48	.76	.74	2.24	.84	.50	.61	.68	.18	.54	.14
68	(.60)	.58	1.99	.66	.39	.48	.46	.16	.36	.13
92	.55	.53	2.08	.75	.27	.43	.44	.19	.35	.10

[1] Figures in parentheses were estimated by interpolation.
[2] Acinar zinc-65 concentration (A_a) was calculated from the following relationship:

$$A_a = \frac{A_p - xA_i}{(1-x)}$$

[3] Islet zinc-65 concentration was calculated from the relationship:

$$A_i = \frac{A_p R}{Rx + (1 - x)}$$

A_i and A_p represent the concentration in islet tissue and whole pancreas, respectively. R is the islet/acinar grain count ratio and x is the fraction of pancreatic tissue occupied by the islet organ.

The calculated islet zinc-65 concentration is recorded in Table 3. For small values of x the calculated islet radioisotope concentration approaches the product of grain count ratio (R) and pancreatic zinc-65 concentration (A_p).

DISCUSSION

Radioactive zinc is widely distributed throughout the rat after intravenous injection and is not preferentially localized in any one organ. There is an initial rapid uptake of radioisotope by the liver, pancreas, intestine, and kidney, but this is quickly excreted or redistributed to other parts of the animal. After the initial adjustments there are only small changes in zinc-65 concentration. The general pattern of distribution in the rat is similar to that reported for mice and dogs by Sheline *et al.* (1943).

A comparison of the studies of the general distribution of radioactive zinc with autoradiographic investigations suggests that the injected zinc passes through the acinar tissue and is excreted into the intestines. During the period of high excretion (the first 24 hours), autoradiograms show that acinar tissue has the greatest relative concentration. Conversely, when excretion reaches a low level, the isotope concentration in acinar tissues is low while that in the islets remains high. Vallee and Neurath (1954) have reported that carboxypeptidase, secreted by the exocrine portion of the pancreas, is a zinc-containing enzyme. Therefore it seems probable that

part, if not all, of the zinc-65 secreted into the intestine may be bound to this enzyme.

By way of contrast the zinc-65 concentration of islet tissue shows remarkably little change. The calculated islet concentrations show no significant differences over the entire period of study (Table 3). It appears that the zinc-65 retained by islet cells is rather firmly bound. Relatively high concentrations of the metal are retained in spite of conditions which might be expected to favor a ready exchange and loss of the metal, i.e., low blood concentration and high vascularity of islet tissue.

The cumulative excretion of zinc-65 (Fig. 1) seems to approach an asymptotic value of considerably less than 100%. An estimate of this asymptote was obtained by finding the value which gave a straight line when log (asymptote-cumulative excretion) was plotted against time (Fig. 2). The asymptote calculated in this manner indicates that about 58% of the injected radiozinc was in a compartment from which excretion was relatively rapid. The biological half-life of this zinc-65 was 40 hours. On the other hand, the remaining 42% of injected isotope had entered a system from which the loss was very slow. Presumably, the islet zinc-65 belongs in this system. Other organs such as blood and skeletal muscle were found to have a similar slow exchange of isotope. It does not necessarily follow that a common metabolic process is involved in all organs showing a similar slow loss; several processes might be involved which together have a long biological half-life.

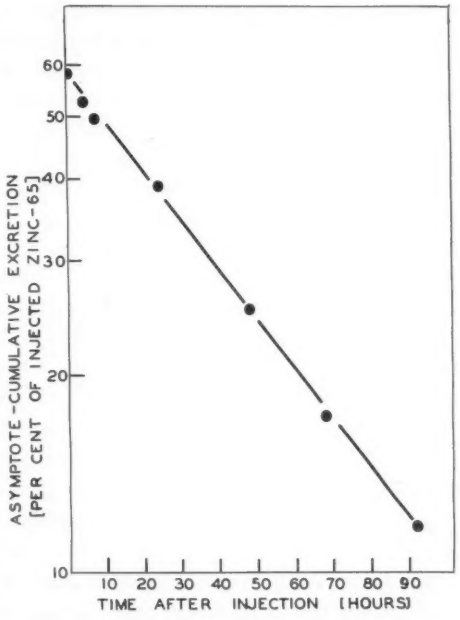

FIG. 2. Analysis of the excretion of zinc-65. The cumulative excretion (urine and feces) appeared to approach an asymptotic value. This asymptote was estimated by finding the value which gave a straight line when log (asymptote-cumulative excretion) was plotted against time. The asymptote was found to be 58% of the injected zinc-65.

The calculated zinc-65 concentration in islet tissue was found to be higher than that of any other organ studied 24 hours and more after injection. The figure of 2.24% of the injected zinc-65 per gm. tissue is about 2.5 times that in the next highest tissue (the liver) and about 3 times that

in the acinar tissue at the 48 hour interval. Weitzel and his coworkers (1953) have shown that the total zinc concentration of the islet organ of 10 species of teleost fish is 5 to 20 times that of the exocrine portion of the pancreas. The amount of zinc in the islet organ of all the species studied was found to be 2 to 9% of the islet insulin content. Although the values reported here for the rat are only approximate, they indicate a similar strong affinity of some islet constituent for zinc.

Attempts to distinguish between the various types of islet cells were unsuccessful. Differential staining was unsatisfactory because so much of the dye was absorbed by the gelatin photographic film that tissue characteristics were obscured. Furthermore, the resolution obtained in the autoradiographs by the technique employed was not sufficient to localize zinc-65 at an intracellular level. The grains appeared to be rather uniformly distributed in the emulsion under the islets. It is known, however, that beta cells comprise the majority of the cells of islet tissue; alpha cells of the rat are arranged principally on the periphery (Gomori, 1943). It seems probable, therefore, that the beta cells were responsible for the major uptake of the isotope.

It is impossible from the data available to appraise the function, if any, of the radiozinc bound in islet tissue. The retention of isotope and its slow loss are similar to that in other organs of the body such as muscle or blood. There is no evidence to suggest that the metal bound in islet tissue is involved specifically in a vital process. Indeed, the metal cobalt was found by Ulrich and Copp (1951) in the pancreas in high concentrations after intravenous administration. Autoradiographs made by these investigators 24 hours after injection showed a diffuse distribution of radioactive cobalt throughout the pancreas, somewhat similar to that reported here for zinc-65 at the same time interval. Thus, localization of zinc in islet tissue may not even be specific but may represent the binding capacity of certain cellular constituents for a group of metals. While the observations reported above demonstrate that zinc accumulates in islet cells and is retained in high concentrations longer in islet than acinar tissue, further studies are needed before any relationship of zinc to islet cell function or insulin production and storage can be postulated.

SUMMARY

An autoradiographic method for studying the retention and distribution of radioactive zinc-65 in the rat pancreas has been described. Zinc-65 entered both islet and acinar tissue, but the rates of loss from these two tissues were strikingly different. Although the acinar cells lost the radioisotope quickly, the concentration in islet cells remained high and constant throughout the 92 hours of observation. The islet zinc-65 concentration was calculated and found to be higher than any organ studied at all periods except those immediately following the injection. Excretion data

indicated that approximately 58 per cent of the injected metal is excreted logarithmically with a biological half-life of about 40 hours. The remainder is retained for a much longer period.

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A COMPARISON OF THE ACTION OF HUMAN AND RAT LIVER SLICES ON SEVERAL CORTICOSTEROIDS[1]

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RECENT studies have demonstrated the great capacity of the rat liver to metabolize adrenal cortical steroids, and, in so doing, to decrease their biological activity (Miller and Axelrod, 1954; Bacchus, 1953; Hechter *et al.*, 1953; Louchart and Jailer, 1952; Paschkis *et al.*, 1951; Schneider and Horstmann, 1951, 1952). There is, however, little information concerning the hepatic metabolism of aldosterone, or the *in vitro* metabolism of adrenal cortical steroids by human liver (Gordon, 1954).

Consequently, it was decided to compare the hepatic metabolism of aldosterone with other adrenal cortical steroids by incubating them with rat and human liver slices, and to conduct these experiments at steroid levels which were closer to the physiological range than those previously used. Finally, in selected cases, an attempt was made to correlate the reduction of Ring A with loss of biological activity following incubation.

MATERIALS AND METHODS

Corticosterone, desoxycorticosterone, and hydrocortisone were incubated with liver slices in amounts of 19 mμM. and 190 mμM. Aldosterone was incubated only at 19 mμM., and cortisone and 9α-fluorohydrocortisone were incubated only at 190 mμM. In order to avoid day to day variation, a few specimens of each steroid were incubated concurrently with slices from the same liver. M/15 phosphate buffer was prepared at pH 7.4 (Peters and Van Slyke, 1932), and then diluted with an equal volume of isotonic saline. Alcoholic solutions of the steroids to be incubated were put into flasks, and dried in air. Two milliliters of buffer were added to each flask.

Young adult male rats of the Slonaker strain were sacrificed by a blow on the head. The liver was rapidly excised, and sliced free-hand. The human liver was obtained by surgical biopsy, and consisted of specimens from eight consecutive, unselected cases. In all cases, approximately 70 mg. of liver was put into each flask. The flasks were incubated for one hour in a water bath at 38° C., and were shaken by hand every 15 minutes during this time. Incubation was conducted in air, since a few experiments failed to show significant differences between results of incubation in air and in 95% oxygen-5% carbon dioxide.

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At the end of one hour the steroid was extracted from the liver and buffer by shaking with redistilled chloroform. The chloroform extract was dried in air; the steroid was dissolved in 95% ethanol, and UV absorption curves were determined in the Beckman DU spectrophotometer. At steroid levels of 190 mμM., macro-cuvettes were used, and the amount of the original steroid remaining was calculated from the extinction coefficient and the optical density at 240 mμ. When 19 mμM. of steroid was incubated, it was necessary to use a micro-attachment and microcuvettes (Pyrocell). Liver blanks were routinely run and were observed to exhibit a small and quite constant UV absorption at 240 mμ. A correction was employed to compensate for this increment.

In order to correlate the loss of biological activity with reduction of Ring A, a bioassay which measures the electrolyte activity of adrenal steroids was used (Johnson, 1954). The results in this bioassay are expressed as the number of micrograms of desoxycorticosterone acetate (DCA) which would produce the observed changes. In order to express this activity in terms of desoxycorticosterone (DOC) and aldosterone (ALDO), the following ratios were used: DOC/DCA = 1.1, and ALDO/DCA = 37 (determined on the same sample of crystalline steroid used in the experiment).

RESULTS

I. *Recovery of steroid*

In order to investigate the adequacy of the extraction procedure, 100 μg. (300 mμM.) of desoxycorticosterone were put into a series of flasks, and the flasks treated in the following manner. Ninety-five per cent redistilled ethanol was added to the first group of flasks, and UV absorption curves determined in the spectrophotometer. This represents the amount of added steroid. The remaining flasks differed in that buffer was added to the second group, and liver and buffer to the third. The steroid was immediately extracted from these flasks by means of the procedure described, and UV absorption curves determined. The results of a typical experiment are illustrated in Figure 1. Since the UV absorption curves of the three groups are virtually identical, chloroform extraction is capable of recovering added steroid from buffer and/or liver. In addition, it can be seen that the liver contributed little to the UV absorption at 240 mμ. in this experiment.

II. *In vitro metabolism of rat liver*

A. *19 mμM. steroid incubated:* The results of incubating 19 mμM. of aldosterone, corticosterone, desoxycorticosterone, and hydrocortisone with surviving rat liver slices are tabulated in Table 1a. Three additional experiments were performed in which only desoxycorticosterone and hydrocortisone were incubated. These results are shown in Table 1b. As can be seen from these results, there is some variation in the total amount of steroid undergoing saturation of Ring A in a given experiment, but the relationships between the steroids are quite constant. Ring A of desoxycorticosterone is by far the most susceptible to hepatic metabolism, that of hydrocortisone the most resistant, and aldosterone and corticosterone seem to be intermediate.

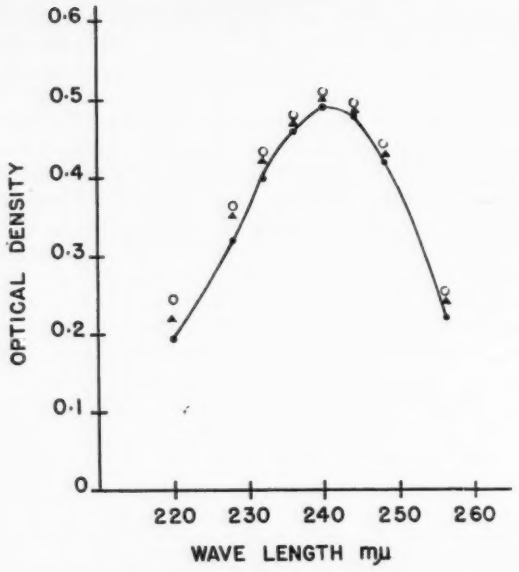

Fig. 1. Ultraviolet absorption spectra of desoxycorticosterone recovered from buffer and/or liver prior to incubation

●—● = desoxycorticosterone standard
▲ ▲ = desoxycorticosterone recovered from buffer
○ ○ = desoxycorticosterone recovered from buffer and liver

FIG. 1

B. *190 mμM. steroid incubated:* The results of incubating 190 mμM. of cortisone, corticosterone, desoxycorticosterone, hydrocortisone, and 9α-fluorohydrocortisone with surviving rat liver slices are tabulated in Table 2a. Three additional experiments were performed in which desoxycorticosterone, hydrocortisone, 9α-fluorohydrocortisone were incubated,

TABLE 1a. 19 mμM. STEROID INCUBATED WITH RAT LIVER

Rat Liver	mμM. of Steroid Recovered			
	Desoxycorticosterone	Corticosterone	Aldosterone	Hydrocortisone
1	11.1	11.5	11.8	12.2
2	11.6	13.0	13.5	15.4
3	11.5	12.7	13.5	14.7
4	10.3	12.4	13.4	15.4
Average	11.1 (58.4%)	12.4 (65.2%)	13.4 (70.5%)	14.9 (78.4%)

TABLE 1b. 19 mμM. STEROID INCUBATED WITH RAT LIVER

Rat Liver	mμM. of Steroid Recovered					
	Desoxycorticosterone			Hydrocortisone		
1	10.9	15.3	11.6	17.5	16.0	16.7
2	12.8	13.5	13.7	17.6	16.5	16.2
3	6.7	10.8	10.0	15.8	14.0	15.0
Average	11.7	(61.6%)		16.1	(84.8%)	

and these results are shown in Table 2b. It is clear that the relationship between corticosterone, desoxycorticosterone, and hydrocortisone that was observed when incubating 19 mμM. of steroid obtained when 190 mμM. was incubated. In addition, cortisone and 9α-fluorohydrocortisone were found to be about as resistant as hydrocortisone.

III. *In vitro metabolism of human liver*

The results of incubating 19 mμM. of aldosterone, corticosterone, desoxycorticosterone, and hydrocortisone with human liver slices are tabulated in Table 3. When using human liver, there was a much greater variation in the total amount of steroid reduced by a given liver, but once again, the relationship between the four steroids was uniform. It also appears that human liver slices metabolize adrenal cortical steroids at a more rapid rate than rat liver.

TABLE 2a. 190 mμM. STEROID INCUBATED WITH RAT LIVER

Rat Liver	mμM. of Steroid Recovered							
	Desoxycorticosterone		Corticosterone	Cortisone		9α-Fluorohydrocortisone		Hydrocortisone
1	136	133	146	149	150	149	146	158
2	139	141	147	159	156	158	159	157
3	133	124	142	152	148	150	153	156
4	124	133	139	146	149	146	149	152
Average	133	(70%)	144 (76%)	152	(80%)	152	(80%)	156 (82%)

TABLE 2b. 190 mμM. STEROID INCUBATED WITH RAT LIVER

Rat Liver	mμM. of Steroid Recovered							
	Desoxycorticosterone		Hydrocortisone				9α-Fluorohydrocortisone	
1	110	114	144	147	145	140	135	138
2	126	124	158	159	161	150	157	162
3	115	112	150	152			147	156
Average	116	(61%)	152	(80%)			150	(79%)

TABLE 3. 19 mμM. OF STEROID INCUBATED WITH HUMAN LIVER

Patient	mμM. of Steroid Recovered			
	Desoxycortico-sterone	Corticosterone	Aldosterone	Hydrocortisone
Y.D.	5.4	—	6.2	9.0
D.T.	8.9	9.1	11.7	15.1
E.K.	5.3	7.0	8.1	9.6
L.R.	10.4	11.5	—	13.7
H.F.	2.9	8.5	9.2	11.7
E.H.	6.5	8.4	8.6	11.6
J.R.	9.6	13.1	14.4	15.9
H.W.	10.9	14.7	13.0	15.6
Average	7.5 (39.5%)	10.3 (54.2%)	10.2 (53.7%)	12.7 (66.8%)

IV. *Effect of incubation on biological activity*

Table 4 compares the mineralo-corticoid activity and UV absorption of desoxycorticosterone and aldosterone following incubation with rat liver slices. In these experiments, the reduction of the Δ4-3-keto configuration of aldosterone and desoxycorticosterone following incubation with rat liver gave a good approximation of the loss of biological activity.

TABLE 4. BIOASSAY OF DESOXYCORTICOSTERONE AND ALDOSTERONE AFTER INCUBATION WITH RAT LIVER

	Dose per rat, estimated on basis of UV absorption (μg.)	Bioassay: Effect on urine K/Na ratio Equivalent to	
		Desoxycorticosterone (μg.)	Aldosterone (μg.)
Desoxycortisoterone	7.9 7.9	5.3±2.0 7.1±2.4	— —
Aldosterone	0.19 0.19	— —	0.22±0.06 0.19±0.06

DISCUSSION

These data indicate that under the conditions of the present experiments there were distinct differences in the rate of uptake and metabolism of various adrenal cortical steroids by rat liver slices. The same relationships were observed with human liver slices. In addition, in the case of aldosterone and desoxycorticosterone, there was a very close correlation between the reduction of Ring A and the loss of biological activity following incubation with rat liver slices.

Contrary to the results of the present experiment in which desoxycorticosterone was the most sensitive, Schneider and Horstmann (1952) have found it to be the most resistant to metabolism by rat liver slices. The possibility of error in the present experiment seems unlikely in view of the

consistency of the results. The most likely explanation for the observed discrepancy is the relative insolubility of desoxycorticosterone. In the present experiment 19 mμM. (6.4 μg.) and 190 mμM.(64 μg.) of desoxycorticosterone were dissolved in 2 ml. of buffer, while Schneider and Horstmann dissolved between 600–900 μg. in 3 ml. of buffer. These authors have previously demonstrated that when the initial concentration of desoxycorticosterone was decreased from 500 to 250 μg. per ml. of buffer, a larger proportion of desoxycorticosterone was metabolized (Schneider and Horstmann, 1951, 1952). Another explanation for the observed difference is that Schneider and Horstmann chromatographed their extract after incubation. These authors then measured the amount of steroid not metabolized in any way, while the present experiment measures the unchanged steroid plus any metabolites that still retain the Δ4-3-keto group. In order to explain the difference on this basis, we would have to assume that in the present experiment over one-half of the material recovered after incubation of hydrocortisone consisted of these metabolites. However, both liver perfusion and incubation studies indicate that only small amounts of metabolites are formed which retain the Δ4-3-keto group (Miller and Axelrod, 1954; Hechter *et al.*, 1953; Schneider, 1952).

In general, when we compare the degree to which liver slices metabolize the Δ4-3-keto group of adrenal cortical steroids, the differences between these compounds seem to be in order of their degree of oxygenation. The only exception to this general rule is aldosterone, which although it possesses five oxygen atoms, is seen to behave more like corticosterone (four oxygen atoms), than like hydrocortisone, which also has five oxygen atoms.

In addition, a few of the specific results merit closer attention. It has been shown that the introduction of a halogen atom on C_9 greatly potentiates both the gluco-corticoid and the mineralo-corticoid activity of cortisone and hydrocortisone (Fried and Sabo, 1954; Borman *et al.*, 1954; Axelrad *et al.*, 1954). For this reason, hydrocortisone was compared with 9α-fluorohydrocortisone. The results indicate that the introduction of a fluorine atom on C_9 did not change the ability of the conjugated unsaturated system of hydrocortisone to withstand hepatic inactivation. It is also interesting to note that desoxycorticosterone, which is relatively ineffective via the oral route, is also the steroid which undergoes the greatest reduction of Ring A as a result of hepatic metabolism.

It appears that the liver plays a major role in the metabolism of adrenal cortical steroids (Tomizawa *et al.*, 1954; Schneider and Horstmann, 1952; Nelson and Harding, 1952). Tyler *et al.* (1954) and Brown *et al.* (1954) have suggested that the removal of circulating hydrocortisone is diminished following surgery and liver disease. A similar defect of hepatic metabolism might be responsible for increased amounts of aldosterone in the urine of patients with hepatic cirrhosis (Luetscher and Johnson, 1954; Chart and Shipley, 1953). Patients with cardiac failure and nephrosis

may excrete excessive amounts of aldosterone without showing clear evidence of hepatic disease or dysfunction. The present experiments have demonstrated that when the amount of steroid available to the liver was diminished ten-fold, the steroid metabolized decreased proportionately. It seems possible, therefore, that reduced hepatic blood flow might limit the rate of metabolism and result in increased levels of steroid.

CONCLUSIONS

Human and rat liver slices reduce the Δ4-3-keto group of aldosterone, corticosterone, desoxycorticosterone, and hydrocortisone.

There are uniform differences in the rate of metabolism of adrenal cortical steroids by human and rat liver. Of the steroids tested, desoxycorticosterone was the most sensitive, hydrocortisone the most resistant, and aldosterone and corticostérone were intermediate. Cortisone and 9α-fluorohydrocortisone, when incubated with rat liver slices, were found to be about as resistant to hepatic metabolism as hydrocortisone.

The electrolyte activity of desoxycorticosterone and aldosterone remaining following incubation with liver was approximately equal to the amount of steroid with unaltered Ring A.

The possible role of the liver in controlling the circulating level of steroids was discussed.

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REGIONAL DIFFERENCES OF THE PANCREATIC ISLET[1]

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SIMARD (1945) found an almost complete disappearance of the A cell in a subcutaneous pancreatic transplant of one dog which had been operated upon following the method of Ivy and Farrell (1926). Our interest in the A cell in relation to Glucagon led us to investigate the possibilities offered by Simard's work.

In the present study we have confirmed Simard's observations that the transplanted segment was devoid of A cells and have concluded that regional differences in islet cell composition were responsible for the absence of A cells in the transplants.

MATERIAL AND METHODS

Eight dogs weighing between 20 and 50 pounds and two mature cats were used. Animals were killed with nembutal and their pancreas completely removed. All tissues unless otherwise specified, were fixed in Zenker-Formol, embedded in paraffin, and cut at 2.5 μ. Some sections were stained with Masson's trichrome and Gomori's Chrome Alum hematoxylin following a procedure previously described (Bencosme, 1952); other sections were stained with Gomori's aldehyde fuchsin stain (Gomori, 1950), or the Mallory-Heidenhain azan stain (Thomas, 1937).

In the first group of dogs (5 animals), the entire pancreas was bisected in the longitudinal axis early during fixation, these two portions were then cut transversally in segments of about 6–7 cm., in length, in such a way that the pancreas could be reconstructed from the paraffin sections in its entire length.

In the second group of dogs (3 animals), the processus uncinatus was cut serially after fixation (about 12–14 sections). These serial sections were cut transversally to a thickness of 3 mm. The body and the splenic area were cut sagitally, as in the first group of dogs thus providing a few large sections. Of these three dogs, one was fixed in formol 10% for twenty days and then processed and embedded in paraffin.

The entire pancreas of the two cats were treated as the pancreas of the first group of dogs.

RESULTS

Two regions in the dog pancreas were clearly distinguishable; a region devoid of A cells (Fig. 3), Zone B corresponding roughly to the uncinatus process; and the remainder Zone A with islets similar to those described in the dog (Thomas, 1937; Gomori, 1939), and composed of A, B and D

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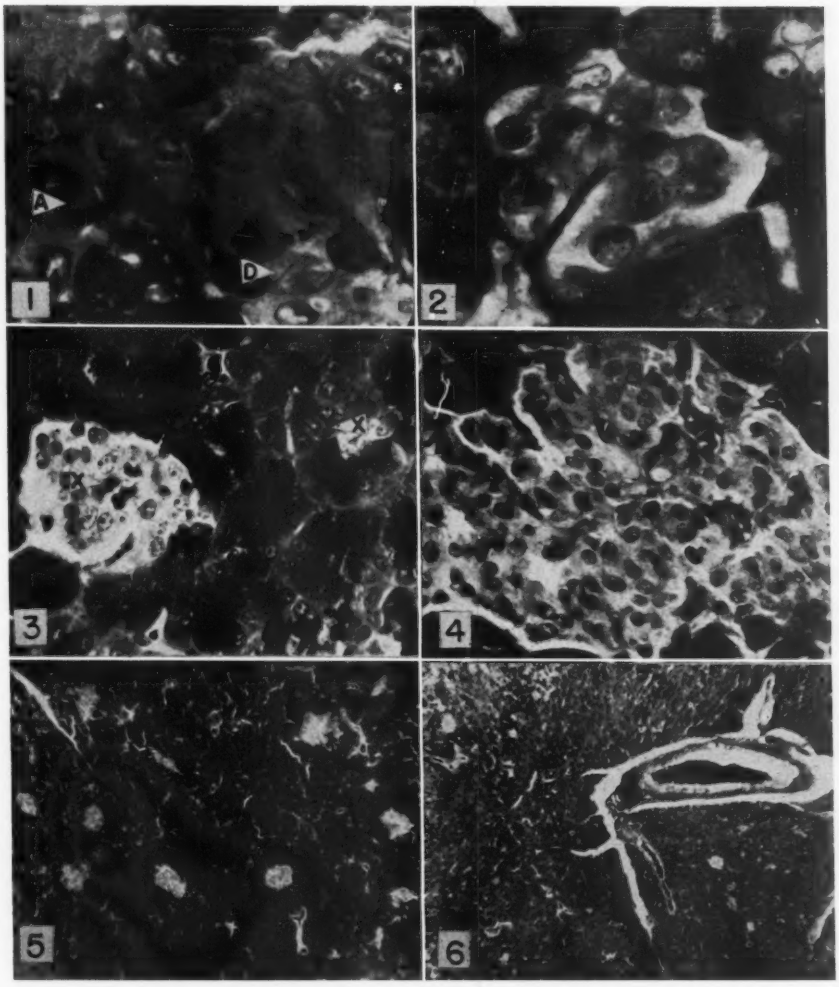

FIG. 1. Zone A of dog pancreas showing A, B and D cells in a pancreatic islet. Masson's trichrome ×950.

FIG. 2. Pancreatic islet without A cell from a transplanted processus uncinatus (Zone B). Masson's trichrome ×950.

FIG. 3. Pancreatic islets of Zone B without A cell. X cells are seen in the islet and in the Acinar tissue. Gomori's Chrome Alum ×400.

FIG. 4. Pancreatic islets of Zone A. Gomori's Chrome Alum ×400.

FIG. 5. General appearance of Zone A. Note large size of islets when compared with those of Zone B as seen in Fig. 6. ×35.

FIG. 6. General appearance of Zone B. Note the small size of the islets. ×35.

cells (Fig. 4). The transition between the two Zones was relatively abrupt. It was also noticed that certain cells present inside islets which displayed a peculiar staining reaction were considerably more numerous in Zone B.

Because of our uncertainty at present as to the exact nature of these cells, they are being referred to in this paper, as X cells (Fig. 3). A summary of the cytological and staining characteristics which differentiate the X cells from the A, B and D islet cells is given in Table 1.

TABLE 1

	A cell Fine granular cytoplasm	B cell Fine granular cytoplasm	D cell Agranular cytoplasm	X cell Coarse granular cytoplasm
Gomori's Chrome Hematoxylin	Red	Steel grey blue	Pale grey	Brown
Gomori's Aldehyde Fuchsin	Red	Purple	Pale green	Pale green
Masson's Trichrome	Grey-black	Pink	Sky blue	Red
Mallory-Heidenhain Azan	Red	Pink	Sky blue	Red

It must be noted that the A granules are only stained brown-black with the Masson's trichrome if the iron hematoxylin is well ripened. Otherwise, the A cell will appear bright red, similar to the X cell. One simple way of obtaining a well ripened hematoxylin consists of leaving the fresh solution in a paraffin oven for a few days until it becomes black and covered with a film which has a metallic reflection.

The X cell was present in Zone B not only in islets but also in the wall of ducts (small, medium and large). Very often the X cell was found forming part of the wall of an acinus (Fig. 3). The islets of Zone B were characteristically smaller in number and size (Figs. 5, 6), contained well granulated B cells and were proportionally richer in D cells than those of Zone A. In Zone A, it was exceedingly rare to find X cells inside islets.

In general, the histological reactions of islet cells was the same for tissues fixed in Zenker-Formol or in Formol. The X cell cytoplasm however stained rather poorly after Formol fixation, and often appeared markedly vacuolated. Formol fixation was nevertheless perfectly adequate to distinguish the presence of A cells in Zone A and their absence in Zone B.

CAT PANCREAS

The findings in the cat's pancreas were similar to those observed is the pancreas of the dog. Zone B in the cat also corresponded roughly to the processus uncinatus, and as in the dog, Zone B showed fewer and smaller islets devoid of A cells but with a large number of D cells. The X cell in the cat seemed to be more uniformly distributed throughout the pancreas.

DISCUSSION

The absence of A cells in the processus uncinatus developmentally related to the ventral pancreas and the presence of A cells in the body and tail of the pancreas developmentally related to the dorsal anlage supports the view that there is a difference between the potentialities of these two anlages. Among the many investigations made possible by this observation is that of determining the glucagon content of a pancreatic tissue devoid of A cells without administering any drug to the animal.

Early in the century, discussions about the differences between the dorsal and ventral pancreatic anlage were mostly concerned with the question of whether the islets were formed exclusively by the dorsal pancreas. An excellent critical review on this subject was written by Bargman (1939), who concluded that it was not proven that islets were only derived from the dorsal pancreas. From the literature it is apparent, however, that there is some difference between the ventral and dorsal pancreatic anlage in relation to their potentiality to form islets (Gianelli, 1909; Clara, 1924; Siwe, 1926; Wolff-Heidegger, 1936; Villamil, 1942; Hard, 1944; Bencosme, 1954).

Differences in the capacities of the ventral and dorsal pancreas to produce A and B cells was suggested by Sergeyeva (1947, 1952) who through a series of duct ligation experiments in young pups and kittens concluded that after ligation two areas could be distinguished in the pancreas, the Alpha and Beta areas. The ligatures used by this author divided the pancreas in such a way that topographically her Alpha and Beta cell areas correspond roughly to our A and B Zones respectively. Sergeyeva (1947 1952), considered her Alpha and Beta areas as projection areas from the dorsal and ventral pancreatic primordia respectively. On the basis of her work (Sergeyeva, 1940, 1943) and that of others (Babkin, 1944; Kuntz, 1945) dealing with the function of the pancreatic nerves, Sergeyeva (1947) suggested that a difference in the innervation of these two areas of the pancreas was an important factor responsible for the different developmental potentialities of the dorsal and ventral pancreas. This author thought that the dorsal pancreas was richer in A cells because its innervation was predominantly sympathetic. It is unfortunate that Richins (1945) in his work on the innervation of the pancreas did not distinguish between the various portions of the pancreas.

In his studies on the neuro-insular complex, Simard (1945) reported that the A cells disappeared almost completely from a subcutaneous pancreas transplant which was totally deprived of its extrinsic innervation. From this work, Simard concluded that normal differentiation and functioning of the A cell was probably under the direct influence of the extrinsic pancreas innervation. He therefore concluded that the lack of such stimulus in the pancreatic transplant was the cause of the disappearance

of the A cells. Topographically, the portion of pancreas transplanted in Simard's work corresponds roughly to our Zone B which is normally completely devoid of A cells.

The present work supports the view that there is a difference between the developmental potentialities of the dorsal and ventral pancreatic anlage although it does not suggest the cause of this difference. Both pancreatic anlages appear to be capable of forming pancreatic islets. However, the islets produced by the ventral pancreas seem to be fewer, smaller and devoid of A cells. This observation provides new interpretation for the results obtained by Simard (1945) and Sergeyeva (1947, 1952). Our work stresses the necessity for regional control in morphological studies of the pancreatic islets as well as the difficulties in interpreting pancreatic biopsies when one is unaware of the topographic differences present throughout the pancreas.

Some authors have mentioned the presence in the pancreas of some cells which probably are identical or closely related to what is here described as the X cell (Mankowski, 1902; Bayley, 1937; Sergeyeva, 1940; Gomori, 1941; Thomas, 1942). Several names and interpretations have been given to these cells. Mankowski (1902) thought it to be a transitional form between acinar and islet cells. This was also the impression of Sergeyeva (1940) who called them "transitional" cells. Gomori (1941) however, considered the Mankowski cell together with some other degenerating forms of the acinar cell.

To our knowledge the Mankowski cell has not been described as a constant finding inside islets. Moreover, the cell here called X cell is found in Zone B on the wall of small ducts just as frequently if not more than inside acinar cavities. In view of the above observations we believe that a reinvestigation of the group of cells which have been described as "Mankowski cells" by Gomori (1943), "granular cells of the acini" by Bayley (1937), "precursor" and "transitional" cells by Sergeyeva (1940), should be made in order to explain their relationship if any with the X cell found inside islets, particularly in those of the uncinatus process.

SUMMARY

From the present work it is apparent that in the mature dog and most likely also in the cat, the portions of pancreas which are derived embryologically from the ventral (processus uncinatus) and the dorsal (body and tail) pancreatic anlage are distinctly different with respect to the cellular composition of their islet tissue. The most marked morphological differences between these two embryologically different pancreatic areas is the lack of A cell in the zone derived from the ventral pancreas in contrast with that derived from the dorsal one.

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AN IMPROVED METHOD FOR ASSAYING THE STEROIDOGENIC POTENCY OF ACTH

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IN A previous communication (Liddle, Island, Cornfield and Forsham, 1954) a new method of assaying corticotropin (ACTH) was described, in which ACTH was injected subcutaneously into guinea pigs and its effectiveness was assessed by measuring urinary steroids of adrenal origin (17-hydroxycorticoids). Consistent agreement was found between the results of this assay method and the results of similar assays performed in normal human subjects. Assay methods which entail the *intravenous* administration of ACTH do not provide information regarding the degree of susceptibility of ACTH to inactivation by extravascular tissues. As a result large discrepancies have sometimes appeared between the labelled potency (based on intravenous tests) and the clinical effectiveness of ACTH when injected extravascularly. The present method avoids such discrepancies.

The main difficulties with the new assay method, as originally described, were that the steroid analyses required unusually careful technique and that the treatment schedule for a complete assay extended over a period of eight weeks or more. It was anticipated that the precision and economy of the method could be improved by (a) use of a shorter period of treatment at each ACTH dosage level and (b) substitution of a less demanding steroid technique. The present communication describes several useful modifications of the original procedure. By employing these modifications it is possible to complete assays in one-twelfth the time previously required; the size of the guinea pig colony may be correspondingly smaller. For a given expenditure of working time the revised method yields more than four times as much information as the original method.

ASSAY PROCEDURE

Male guinea pigs weighing between 300 and 600 gm. are used. The animals are maintained on a diet of fresh cabbage with additional feedings of rabbit pellets between urine collection periods. A crossover design is utilized in carrying out assays, so that each dose of each preparation to be assayed is administered in turn to each animal. Successive treatments may be given on consecutive days. Appropriate doses of ACTH are dissolved in physiologic saline and injected subcutaneously. Urine is collected for a standard

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period of at least seven hours following treatment. Specimens less than 15 ml. in volume are rejected.

Steroid Determinations: The excretion of unconjugated 17-hydroxycorticoids is determined by the following modification of the method of Silber and Porter (1954).

Reagents:

(a) *Methylene Chloride*—This solvent is purified by passing through a bed of silica gel (average 100 mesh) in a 7×125 cm. column. Ten to twenty liters may be purified in 2–3 hours. The effectiveness of purification is determined by running the reaction with the phenylhydrazine-sulfuric acid-ethanol reagent (f). No visible color should develop on standing overnight at room temperature. The solvent keeps for many months at room temperature.

(b) *0. 1 N Sodium Hydroxide*

(c) *70% Sulfuric Acid*—700 ml. C.P. grade sulfuric acid added to 300 ml. H_2O.

(d) *Ethanol*—This is purified by adding 7 gm. silver nitrate and 15 gm. potassium hydroxide separately (each dissolved in 500 ml. ethanol) to 4 liters of absolute ethanol. This is mixed and allowed to stand overnight. This is distilled, using a Vigreux column. The first 700 ml. and the last 100 ml. portions are discarded. The effectiveness of purification is determined by adding this ethanol to the phenylhydrazine-sulfuric acid (f). No visible color should develop on standing overnight at room temperature.

(e) *Blank Reagent*— Two parts (c) with one part (d).

(f) *Phenylhydrazine-Sulfuric Acid-Ethanol Reagent*—100 mg. of phenylhydrazine hydrochloride (recrystallized from ethanol and water) is dissolved in 100 ml. of (e).

(g) *Hydrocortisone Standard*—Ten mg. of hydrocortisone dissolved in 100 ml. ethanol.

Procedure:[1] A 5 ml. aliquot of urine is extracted by shaking for 20 seconds with 20 volumes of methylene chloride in a 100 ml. ground glass stoppered cylinder. After standing 10 minutes the upper (aqueous) layer is discarded. The extract is washed by shaking with 1/20 volume of 0.1 *N* NaOH for 20 seconds. After standing 10 minutes the alkali layer is discarded. Two 40 ml. aliquots of the methylene chloride extract are taken; to one is added 3 ml. of blank reagent ("e," above); to the other is added 3 ml. of the phenylhydrazine-sulfuric acid reagent ("f," above). The solutions are mixed by shaking for 20 seconds. After standing for 15 minutes, the upper (methylene chloride) layer is discarded by aspiration. The remaining contents of the cylinders are allowed to stand overnight at room temperature. Hydrocortisone standards are subjected to the same extraction and colorimetric procedures used for urine. The optical densities at 410 millimicrons are determined using a Beckman DU spectrophotometer.

The method of calculating the amount of "17-hydroxycorticoids" from the optical densities is similar to that described previously. Statistical methods employed in the original paper are applicable.

RESULTS

The characteristics of the response to ACTH under the conditions outlined above are represented in Figure 1. Preparation #284 which had been "highly purified" by adsorption on oxycellulose (method of Payne, Raben, and Astwood, 1950), was assayed over a 512-fold dosage range. In the mid-portion of the curve there was at least a 16-fold dosage range over which response could be represented as a rectilinear function of log-dose.

[1] This method is quite suitable for the determination of 17-hydroxycorticoids (i.e., 17,21-dihydroxy-20 keto corticosteroids) in human urine provided the conjugated corticoids are first hydrolyzed by incubation with β-glucuronidase.

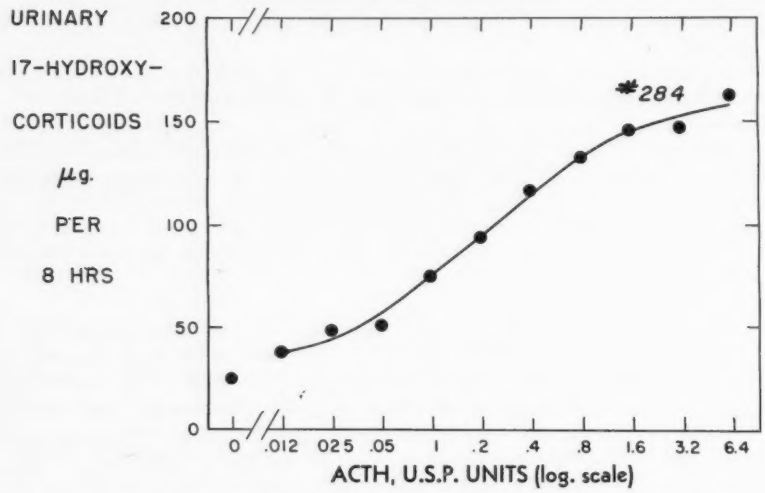

FIG. 1. 17-hydroxycorticoid excretion in response to varying doses of highly purified ACTH. Each dot represents the average response of 12 guinea pigs.

Within this range the index of precision (standard deviation ÷ slope) has been found to be approximately 0.2 to 0.3.

Preparation #284 was assayed against another "highly purified" preparation, #530 (Fig. 2). Although these two materials had similar preparative histories, they still exhibited appreciably different potencies when injected extravascularly and assayed in terms of resulting increases in urinary corticoids. Preparation #284 was, U.S.P. unit for unit, 1.58 (1.38–1.89) (95% confidence limits) times as effective as #530.

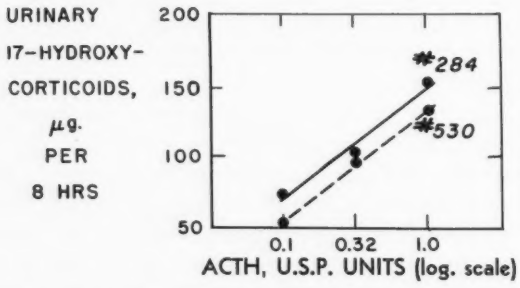

FIG. 2. 17-hydroxycorticoid excretion in response to varying doses of two different ACTH preparations. Each dot represents the average response of 24 guinea pigs. Ratio of potency of #284/#530 = 1.58 (1.38–1.89).

COMMENT

The previously described assay of ACTH in guinea pigs was developed in an effort to simulate in the animal laboratory the circumstances under which ACTH was used clinically. The essential conditions were that ACTH should be injected extravascularly and that the activity of ACTH should be measured in terms of steroidogenesis. It was shown that when these principles were followed there was very close agreement between estimates of potency based upon the assay in guinea pigs and estimates based upon similar assays in human subjects. This assay method, however, was relatively slow and technically exacting. These disadvantages have been overcome to a great extent by the following innovations. Pre-ACTH control determinations are no longer made. Steroid determinations are no longer run in duplicate. Variations in "reagent blank" values contribute so little to the over-all error of the present method that it is sound economy not to determine these values routinely but instead, to use the equivalent time in performing additional animal tests. A single injection of ACTH in aqueous vehicle is now used instead of three injections of ACTH in gelatin. The urine collection is now limited to 8 hours instead of 48 hours. Animals are now tested at intervals of one day instead of two weeks. During test periods a diet of cabbage (rather than rabbit pellets) is used, and this results in adequate urine volume and substantially reduces the "blank" reading due to non-steroidal chromogens. Methylene chloride, rather than butanol, is used to extract steroids from the urine, and this increases the precision and simplifies the steroid analysis itself. A solvent more polar than methylene chloride is not required because, as demonstrated by Burstein and Dorfman (1954), "free" hydrocortisone appears in large quantities in the urine of guinea pigs.

With the faster and more reliable technique now employed, duplicate determinations, pre-ACTH control, and frequently "reagent blanks" can be abandoned in favor of multiplying the number of animal tests. It is not difficult, using the present method, for a single competent technician to carry out as many as 240 individual animal tests during the ordinary working week.

SUMMARY

The "extravascular-steroidogenic" method of assaying ACTH has been revised making use of a simplified technique of steroid analysis and an accelerated treatment schedule. Use of methylene chloride (rather than butanol) to extract Porter-Silber reacting steroids from urine has resulted in increased precision as well as simplicity. By employing single injections of ACTH in saline (rather than multiple injections of ACTH in gelatin) it is possible to complete an assay in 6 days, whereas formerly 12 weeks

were required. These modifications have resulted in a reduction of over 75% in the time and expense required to obtain a given amount of information.

Acknowledgments

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HUMORAL HYPOTHALAMIC CONTROL OF ANTERIOR PITUITARY: A STUDY WITH COMBINED TISSUE CULTURES

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THE current concepts of hypothalamo-pituitary relationships are based on the results of numerous *in vivo* experiments (Harris, 1951; Fortier, 1951; McCann, 1953; Hume, 1949; Benoit and Assenmacher, 1953). The possibility of investigating this problem by simple *in vitro* techniques was particularly challenging in view of a number of findings which suggested the necessity of some hypothalamic factor, so far overlooked, for the release of pituitary hormones *in vitro*. Indeed, conflicting results are to be found in the literature regarding possible secretion of hormones by the various endocrines in tissue cultures. If we consider the anterior lobe of the pituitary, it has been reported as able to produce gonadotrophins (Rosenberg, 1954), or growth hormone (Gaillard, 1948; Martinovitch, 1953) whereas other investigators concluded that it could not secrete gonadotrophins (Cutting and Lewis, 1938) or any hormone whatever (Anderson and Haymaker, 1935). It appears from a study of this literature that, whenever hormonal production has been found at all, it has always been early (4–5 days) after explantation of the tissue; older cultures of the anterior pituitary, though producing a good outgrowth, yield negative results in hormonal assays. To test the hypothesis of the necessity of of a hypothalamic factor for pituitary activation *in vitro*, we therefore decided 1) to study the release of one hormone (ACTH) in cultures of the adenohypophysis as a function of time; 2) to investigate whether the ACTH secretion could be modified by adding to the tissue cultures of the pituitary, fragments of hypothalamic tissue. A simple "direct" assay procedure for hypothalamic hypophysiotropic activity was contemplated along the same lines, should any specific effect due to the hypothalamic tissue be demonstrable.

MATERIALS AND METHODS

Tissue Cultures. Dog and rat tissues have been utilized in various experiments as reported in Tables 1 and 2. The donor rats were males of the Sprague-Dawley strain,

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TABLE 1. ACTH ACTIVITY (SAYERS TEST) IN FLUIDS OF TISSUE CULTURES OF THE ANTERIOR LOBE OF THE PITUITARY

Age of culture fluids tested	Number of rats for each ACTH assay	Variation in ascorbic acid concentration γ/100 mg. adrenal	Degree of statistical significance
4 days*	8	-65 ± 18	$p \leqq 0.02$
4 days†	8	-101 ± 18	$p < 0.01$
8 days*	8	$+22 \pm 22$	
12 days†	7	$+23 \pm 20$	
15 days*	6	-14 ± 5	$p = 0.05$
19 days*	8	-10 ± 11	$p > 0.05$
19 days†	6	$+38 \pm 22$	
26 days*	7	$+61 \pm 9$	
31 days*	6	$+66 \pm 25$	

* dog tissues.
† rat tissues.
Results as arithmetic means and standard errors. Statistical significance calculated by Student "t" factor comparing in each experimental group final to initial values of adrenal ascorbic acid concentrations as obtained in Sayers test.

weighing between 200–250 gm. at time of tissue explantation. The dogs were young male mongrels. The pituitaries of the dogs were removed after the animals had been bled under rapid Nembutal anesthesia; the rats were killed by decapitation and the pituitaries removed immediately. The tissues were grown in plasma clot, according to the standard roller tube method (Parker ,1950; Pomerat, 1951). The fluid bathing the tissues was made up of 50% human ascitic fluid, 45% Earle's solution and 5% chicken embryonic extract

TABLE 2. ACTH ACTIVITY (SAYERS TEST) IN FLUIDS OF COMBINED CULTURES OF THE PITUITARY AND VARIOUS TISSUES

Fluids from combined cultures of	Number of rats for each ACTH assay	Variation in ascorbic acid concentration γ/100 mg. adrenal	Degree of statistical significance
Ant. pituitary + Post. hypothalamus*	8	-94 ± 11	$p \leqq 0.01$
Ant. pituitary + Eminentia media*	7	-188 ± 22	$p < 0.01$
Ant. pituitary + Ant. hypothalamus*	6	$+41 \pm 32$	
Ant. pituitary + Brain cortex*	3	$+28 \pm 15$	
Ant. pituitary + Spleen*	3	-31 ± 25	$p \geqq 0.5$
Ant. pituitary + Liver*	4	$+55 \pm 16$	
Post. hypothalamus† Alone (12 days)	7	$+18 \pm 7$	
Post. hypothalamus* Alone (4 days)	10	-3 ± 10	$p \geqq 0.8$

* dog tissue.
† rat tissue.
See footnote of Table 1.

(EE20 home made or diluted from EE100 of the Difco Laboratories). In the course of this series of experiments ascitic fluids from two different patients with carcinoma were used. The human ascitic fluid was passed through a Seitz bacteria filter and 1,000 units of penicillin G sodium salt, plus 1.50 mg. dihydrostreptomycin sulfate per cc., were added. Two cc. of the culture fluid were introduced per tube and were thereupon removed and changed every 4th or 5th day until the end of the experiment. 19 day old fluid therefore means a fluid which bathed the tissues from day 15 to 19. Usually three explants were placed on each cover slip. Enough tubes were prepared in each experimental group to yield at least 20 cc. of fluid when they were pooled.

Rat and dog pituitary tissue was cultured by the above described method up to 35 days of *in vitro* life. On the 15th or 22nd day after beginning of the pituitary cultures, explants of posterior hypothalamus, anterior hypothalamus and median eminence were introduced for 4 days in some of the pituitary tubes. Fragments of brain cortex, spleen, and liver were used as control for the effect of the hypothalamic tissues. Combined cultures of pituitary and other organs were done by introducing the cover slip bearing the "new" tissue, back to back to the one holding the pituitary explants. All tissues used in combined cultures came from the same species.

The growth of the tissues was checked in the living state under phase microscopy, using still pictures and cine-microphotography. The outgrowths of the various tissues were stained at various phases of the experiments with Azan as modified for tissue culture work (Koneff, 1938). None of the explants of the tissues utilized in the experiments reported here was cut for microscopic examination.

Bioassays. The ACTH activity was assayed in the hypophysectomized male rat (Hormone Assay Laboratories, Chicago) weighing 90–100 gm and hypophysectomized 16–20 hours prior to the test, by using Sayers' adrenal ascorbic acid test. The fluids to be assayed (0.5 cc. or 1 cc.) were injected at time zero in the jugular vein under ether anesthesia.

The fluids of the various tissue cultures were assayed for the presence of the following substances, in the manners indicated:

a. Epinephrine and nor-epinephrine: intra-carotid recording of the blood pressure of the atropinized cat (Hökfelt, 1951), and inhibition of the spontaneous rhythmicity of the isolated rabbit's gut.
b. Histamine: contraction of the guinea pig's gut, subsequently prevented with pyribenzamine ($1/10^6$).
c. 5-hydroxytryptamine (Serotonin): guinea pig's gut, rabbit's gut.
d. Vasopressin: Blood pressure of the rat or the cat injected with the adrenolytic drug SKF-501 (Dekanski, 1952).
e. Oxytocin: guinea pig's uterus.

RESULTS

Table 1 shows that ACTH activity is found in the fluids of the tissue cultures of dog or rat pituitary after 4 days of *in vitro* life. It was not found at later intervals, i.e. 8, 12, 15, 19, 26, and 31 days of culture.

Table 2 shows that introduction of posterior hypothalamus fragments in the pituitary culture tubes from day 15 to 19 (dog tissues) is followed by reappearance of ACTH activity in the cultures' fluids. Identical results were obtained with introduction of explants of median eminence (dog) from day 22 to 26. No ACTH activity was found in similar pituitary cultures when explants of spleen, liver, brain cortex, anterior hypothalamus (dog) were introduced in the roller tubes from day 22 to 26. No ACTH

activity was demonstrable either, in the fluid of cultures of posterior hypothalamus alone for 12 days (rat) or 4 days (dog). Assays for epinephrine, nor-epinephrine, 5-hydroxytryptamine were negative in all fluids, at the level of sensitivity of our methods (epinephrine $\leqq 0.1$–0.2γ; nor-epinephrine $\leqq 0.05$–0.1γ; 5-hydroxytryptamine $\leqq 0.1$–0.2γ). Traces of histamine ($<0.1\gamma$) were occasionally found, especially in the control cultures with liver and spleen tissue. Vasopressor and oxytocic activity were found in the fluids of the cultures where anterior nuclei of the hypothalamus were present (assay sensitivity: vasopressin = 1 mU; oxytocin $\leqq 0.5$ mU).

FIG. 1. Young undifferentiated cells of the outgrowth of a 6 day old tissue culture of the anterior pituitary (Azan, ×190).

The growth of the various explants of anterior lobe of the pituitary was good though not exuberant, possibly due to the small amount of embryonic extract used in our procedure. Differentiation of the cells of the outgrowth started around the 12th day of culture and was accompanied, as the cultures grew older, by accumulation of granules in the cells which were losing their young undifferentiated embryonic appearance (Figs. 1, 2, 3). No cells reminiscent of gonadectomy-, adrenalectomy- or thyroidectomy-cells were seen in any of the stained cultures. Little outgrowth was seen around the various tissues added to the pituitary cultures for 4 days, except in the case of the explants of median eminence which proliferated at a surprisingly fast rate. Upon staining, these proliferating cells of the median eminence

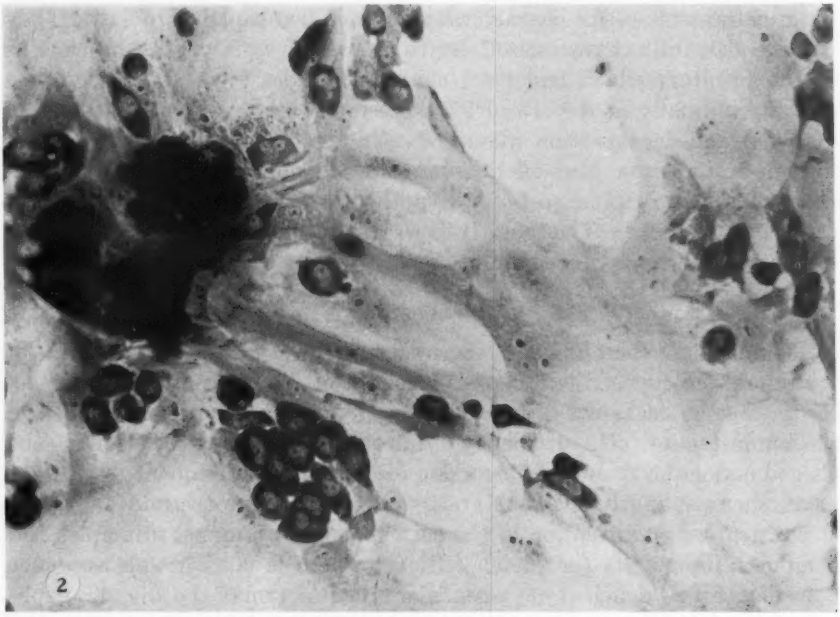

FIG. 2. Numerous differentiated cells in the outgrowth of a 15 day old tissue culture of anterior pituitary (Azan, ×190).

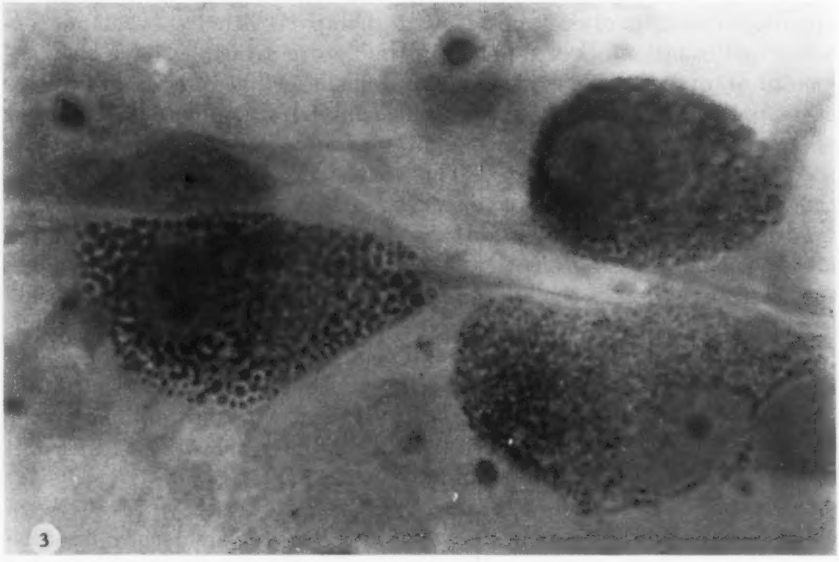

FIG. 3. Three "adult" acidophils with numerous intra cytoplasmic granules in outgrowth of a 35 day old tissue culture of anterior pituitary (Azan, oil immersion). Three chromophobes are in the field but show poorly on the black and white print.

outgrowth assumed the characteristics of spindle chaped fibroblasts. They could not be fully characterized due to their short *in vitro* life. Outgrowths of the pituitary which had been confirmed under phase microscopy, as growing normally at day 15 or 22, were found 4 days later in a state of cytolysis and degeneration when grown in combined culture with spleen, liver or brain cortex. No such degeneration was noted in the combined cultures of pituitary plus posterior hypothalamus, eminentia media or anterior hypothalamus. The tissue lysis was first seen under phase microscopy and later confirmed after staining.

DISCUSSION

The results of these *in vitro* experiments are best interpreted in terms of a humoral (as opposed to a neural) mechanism. To ascertain endocrine activity, the classical rules of endocrinology require three orders of evidence: a) demonstration of a deficiency syndrome following extirpation of the alleged endocrine tissue; b) correction of the deficit subsequent to replacement therapy (auto or homo-graft, administration of crude extracts); c) purification and isolation of the corresponding hormonal principles and demonstration of its (or their) activity. There is considerable evidence nowadays for a "deficiency syndrome" in the function of the adenohypophysis following destruction of the hypothalamus in the otherwise intact animal (Harris, 1951, 1954; Hume, 1949; Ganong and Hume, 1954; McCann, 1953; Anand, 1954). On the other hand, restoration of normal pituitary function after "replacement therapy" with hypothalamic extracts and isolation of corresponding stimulatory principles have been advanced on questionable evidence. The difficulties inherent to both phases of the problem, in working with the whole animal, are numerous for reasons of techniques and perhaps unsurmountable for reasons of logic (cf. Fortier's theory of a dual control for ACTH release; Fortier, 1952). The *in vitro* experiments reported here, have shown: a) that the pituitary "deficiency syndrome" is easily demonstrable; b) that it is corrected by introduction of hypothalamic tissues in the *in vitro* pituitary system. Furthermore, specificity of action of the hypothalamic tissue can be reasonably inferred from the data obtained with control tissues and the bioassays of the various fluids for epinephrine, acetylcholine, histamine, etc. Regarding the role of these various autonomic substances as possible mediators for pituitary ACTH release, the failure to demonstrate them with our methods in the *in vitro* system is in keeping with the conclusions reached by entirely different methods *in vivo* (Guillemin, 1954, 1955).

Such an *in vitro* technique or some modification of it, may prove to be of value as a possible bioassay in the isolation of hypothalamic neuro-humoral substances related to pituitary functions.

The literature on *in vitro* cultivation of nervous cells and nervous tissue is extensive (see bibliography in Murray and Kopech, 1953). There is no doubt that nervous cells, even the highly specialized cells of adult or-

ganisms, can be maintained *in vitro* for any length of time. The recent study with similar techniques of the dynamic changes produced in the cells of the nucleus supra-opticus by hypo- and hyper-tonic solutions (Hild, 1954) is another example of the adequacy of the tissue culture method for elements of the central nervous system.

The "amounts of ACTH activity" found in these experiments, though we never attempted to quantitate our assays, are in agreement with Sayers and Sydnor (1954) if we compare their dilution factors of the whole pituitary in some of their assays and the amount of tissues used in tissue culture techniques. The fact that ACTH was found at all in our conditions (incubation for several days with tissues, at 37.5−38° C., in an absolutely neutral medium) may be more surprising in view of the alleged susceptibility of ACTH to proteolytic enzymes especially at non-acid pH. It may be that the nutrient fluid used in these experiments, known for years by tissue culturists to be the optimal medium, is effective because of its high content in inhibitors of proteolytic ferments.

It was of interest to note the dissociation between morphology and function of the hypophysial outgrowth. Outgrowth from the primary explant and differentiation from the early undifferentiated "fibroblast-like" cells to the classical types of pituitary cells can take place *in vitro* in complete absence of any hypothalamic principle as repeatedly seen by the numerous investigators who over the past 25 years, have grown pituitary *in vitro* (see bibliography in Murray and Kopech, 1953). Interestingly enough, ACTH activity was never found, when the combined cultures with hypothalamic tissue were performed before the 12th to 15th day of the *in vitro* pituitary life, that is, before any differentiated "adult" cells were present in the outgrowth. Similarly, ACTH activity in the fluids was not different from the controls when combined cultures of pituitary and hypothalamus were initiated and studied from day zero to 12 (Guillemin, unpublished).

Another point which was of considerable surprise to us, was the complete absence in pituitary tissue cultures of the vacuolated cells generally related to castration, thyroidectomy, or adrenalectomy. Indeed, when pituitaries of castrated, thyroidectomized or adrenalectomized rats, shown to contain the corresponding "ectomy cells," were placed in tissue cultures, the cells of the outgrowth never showed these characteristics and cells of the explant reverted to normal (Rosenberg, unpublished). It would thus seem that, for their appearing after removal of gonads, adrenals or thyroid the respective "ectomy-cells" need, along with the absence of the peripheral hormone, a constant hypothalamic stimulation. This could not have been properly studied by the particular technique used in this experiment with only 4 days of combined culture with hypothalamus. The data recently reported by Bogdanove and Halmi (1953) on the lack of appearance of thyroidectomy cells in rats treated with propylthiouracyl after hypothalamic lesions, would confirm this hypothesis. Our observations, however, do not clarify the locus of action of the peripheral hormones in their regula-

tory influence on the pituitary which could be trans-hypothalamic or directly hypophysial. Our experiments do not explain either, whether the hypothalamic hypophysiotropic activity is exerted on synthesis of ACTH by the pituitary tissue, on its release or on both phenomena.

The absence of cytolysis of the pituitary outgrowth after combined culture with anterior hypothalamus, posterior hypothalamus and median eminence is remarkable in view of the constancy of the pituitary tissue lysis when brain cortex, spleen or liver were cultured along with it. As mentioned before, this tissue lysis was first noticed under phase microscopy and subsequently confirmed by staining. Possible artifacts due to a bad fixation, for instance, are therefore eliminated. It is tempting to assume that this structural and ecological compatibility of hypothalamic and pituitary tissues might be correlated with their physiological interrelationship (Blount, 1930). The same compatibility has been reported with the tissue of the posterior lobe of the pituitary (Gaillard, 1937).

We did not attempt in these experiments to elucidate the role of vasopressin in the release of ACTH by the pars distalis, a role recently given considerable importance by various investigators (Mirsky *et al.*, 1953; McCann and Brobeck, 1954). But the latter school of thought will have to account for the fact that ACTH was released in a system in which no posterior lobe activity was demonstrated (by assays for vasopressor and oxytocic hormones) and no ACTH was found when vasopressor or oxytocic activity was demonstrated in the fluid of combined cultures of pars distalis and nuclei of anterior hypothalamus. We have already presented further evidence of this fact when pure arginine-vasopressin, kindly given by Dr. V. du Vigneaud, did not release ACTH in the *in vitro* pituitary (Guillemin, 1955; Guillemin and Hearn, 1955).

SUMMARY

Dog and rat anterior pituitary has been cultured in roller tubes. ACTH activity in the fluid medium (Sayers test) was found only in the first 4 day old sample. No ACTH activity was demonstrable in 8, 12, 15, 19, 22, 31 day old samples in spite of excellent growth and differentiation of the pituitary outgrowth. When explants of hypothalamus or median eminence were added to the pituitary cultures from day 15 to 19 or 22 to 26, ACTH activity was re-initiated. No ACTH activity was found in control hypothalamus cultures nor when pituitary was grown from day 15 to 19 or 22 to 26 with explants of brain cortex, spleen, liver. At the level of sensitivity of the bioassays utilized, histamine, acetylcholine, adrenaline, nor-adrenaline, serotonin, oxytocin and vasopressin could not be demonstrated in the fluids of combined cultures of pituitary plus hypothalamus where ACTH activity was found. It is postulated that there exists some hypothalamic hypophysiotropic mediator involved in ACTH release, which is different from the above mentioned substances. The *in vitro* pituitary is proposed as an ideal bioassay for study of hypothalamic hypophysiotropic principles.

Acknowledgments

We wish to express our particular gratitude to Dr. C. M. Pomerat, Professor of Cytology, University of Texas Medical Branch, Galveston, Texas, for his interest in this project and his help, since in our early experiments the tissue cultures were performed in his laboratory in Galveston.

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HISTOLOGICAL OBSERVATIONS ON THE ZONA GLOMERULOSA OF THE RAT ADRENAL CORTEX FOLLOWING ADMINISTRATION OF CORTICOTROPIN PREPARATIONS

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THE zona glomerulosa of the adrenal gland of the hypophysectomized rat maintains its morphological integrity and a readily demonstrable lipid content, while the zonae fasciculata and reticularis atrophy (Deane and Greep, 1946; Greep and Deane, 1949). These observations have suggested that the zona glomerulosa of the rat adrenal cortex is independent of the pituitary gland (Greep and Deane, 1949; Yoffey and Baxter, 1949). The results of other investigators have suggested, however, that the zone may not be entirely autonomous. In mice (Jones, 1949) and in rats (Cater and Stack-Dunne, 1953; Wexler *et al.*, 1955) marked activity in the zona glomerulosa has been observed following the administration of corticotropic fractions. Morphological alterations of the zone in various human disease states have been reported by Stephens (1940), Conn (1949) and O'Donnell *et al.* (1951), while Landing and Feriozi (1954) note that under certain conditions, it may be responsive to ACTH.

The experiments reported here were designed to test a hypothesis based on observations incidental to earlier experimentation, i.e., lipid mobility in the zona glomerulosa of the rat is subject, in part at least, to pituitary control. We find that some, but not all, ACTH preparations may produce exhaustive depletion of sudanophilic material in the zona glomerulosa.

MATERIALS AND METHODS

Hypophysectomized male Sprague-Dawley rats (160–180 gm.) were used throughout these experiments. Injections were begun 24 hours postoperatively. The experiments were carried out in a temperature controlled room at 23°C. All animals were given a regular commercial diet (Purina) and drinking water *ad libitum*. Twenty control animals, also hypophysectomized, were given 0.1 ml. physiological saline in single, daily, subcutaneous injections through 21 days. Survivors were sacrificed 24 hours after the last injection. Sixteen groups of ten rats each received ACTH in one of the following dose increments: 0.33, 0.67, 1.0 and 3.0 units (U. S. P.) per 100 grams body weight. Two brands

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of ACTH* were used. Each brand was used in two forms, aqueous solution and gelatin suspension. The four preparations were injected at each of the above dose levels. As with controls, single daily, subcutaneous injections were given through 21 days. Animals dying before 21 days were examined immediately post mortem. Obviously moribund rats were sacrificed. Animals dying at a time inconvenient for immediate examination were discarded.

Adrenal glands removed for histological analysis were fixed in 10% buffered neutral formalin (Lillie), embedded in gelatin and cut at 6–10 micra on the freezing microtome. The sections were stained with sudan orange and hematoxylin or with sudan black B and carmalum.

RESULTS

Control Series: The adrenal glands of the saline-injected animals showed proliferation of glomerulosa cells and retention of lipid in the cells which

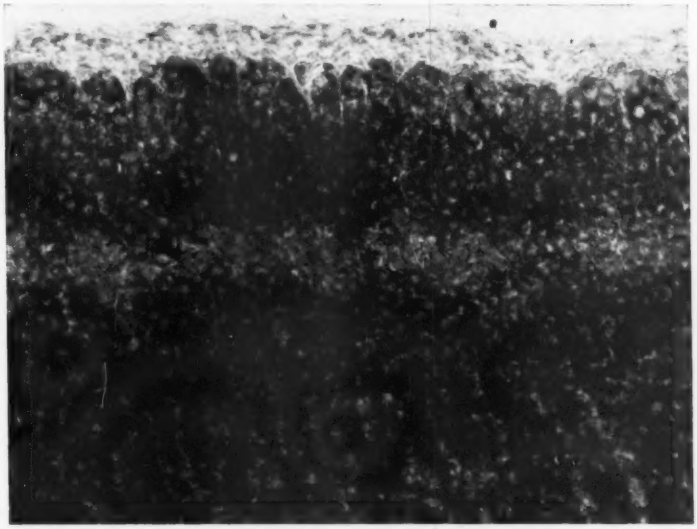

FIG. 1. Zona glomerulosa of Sprague-Dawley rat (♂), 1 day post-hypophysectomy, saline treated, subcutaneously, single injection. Zona glomerulosa appears normal. Lipid-free zone apparent. (Sudan Orange, ×270)

had been present in the zone prior to hypophysectomy (Figs. 1, 2, 3 and 4). As the animals died, or were sacrificed in the moribund state, the lipid-free (sudanophobic) zone, located between the zona glomerulosa and the zona fasciculata, appeared to increase in width with time, being greatest at the end of the experimental period (21 days). The zona fasciculata and reticularis became atrophic (compare Figs. 2 and 3) with characteristic alteration in the physical nature of the individual lipid droplets (from fine coalescence to punctate granularity). This change was attended by gradual

* The ACTH preparations manufactured by the Armour Laboratories and The Wilson Laboratories, were purchased at local outlets.

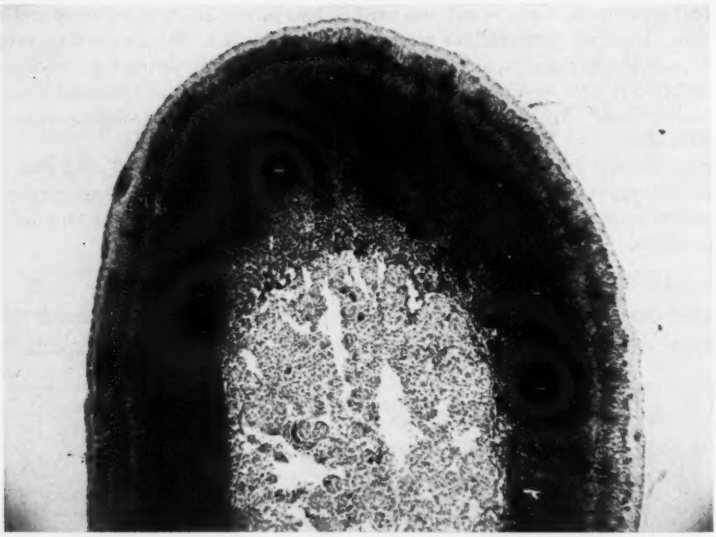

FIG. 2. Cortex. 5 days post-hypophysectomy, saline treated, subcutaneously once daily. Lipids undisturbed in zona glomerulosa. Compare width of lipid-free zone with Fig. 3 (Sudan Orange, ×60)

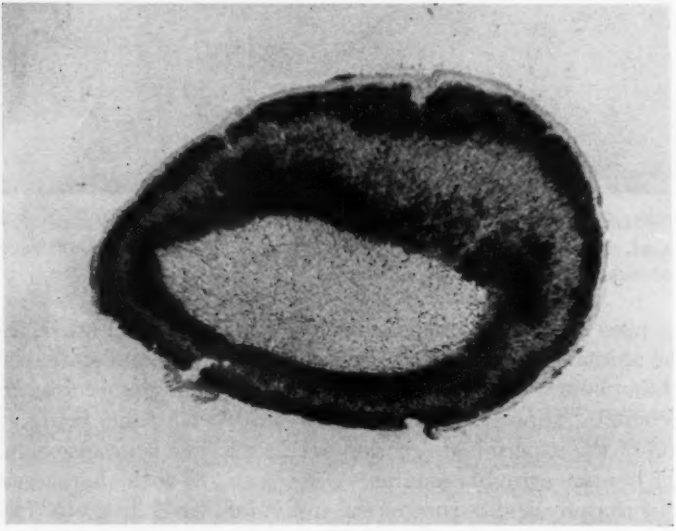

FIG. 3. Cortex. 21 days post-hypophysectomy, saline treated, subcutaneously, once daily. Lipids inert and zona glomerulosa maximally developed. Note distention of lipid-free zone and extensive atrophy of zona fasciculata. (Sudan Black, ×35)

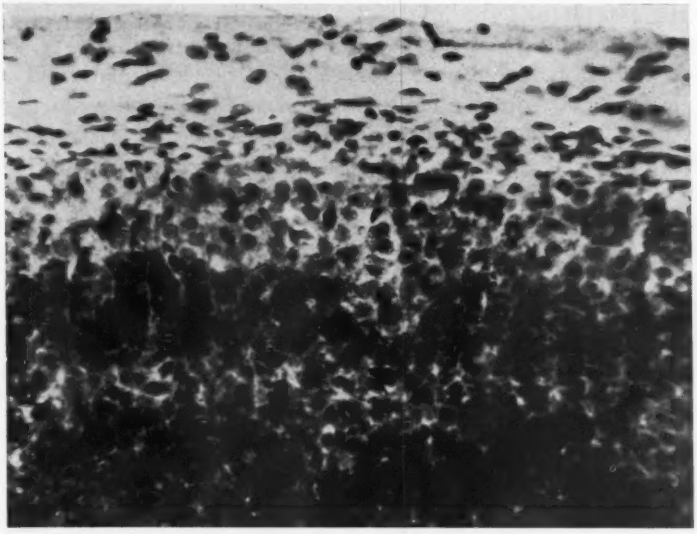

FIG. 4. Zona glomerulosa. Saline treated, nine days post-hypophysectomy. Hyperplasia of cells between capsule and lipid-rich pre-hypophysectomy cells. A slight amount of lipid may be seen occasionally in the newly proliferated cells. (Sudan Orange, ×270)

diminution of lipid from these zones. The lipid in the zona glomerulosa, in contrast, was retained, although in the seven animals examined before day 21 it appeared increasingly granular as the experiment progressed. During this time the cells, newly proliferated following hypophysectomy, lying be-

TABLE 1. INCIDENCE OF LIPID DEPLETION IN THE ADRENAL ZONA GLOMERULOSA OF HYPOPHYSECTOMIZED MALE, SPRAGUE-DAWLEY RATS TREATED WITH ACTH PREPARATIONS IN SALINE AND GELATIN MENSTRUA

Daily Dose in USP Units	ACTH A Aqueous (Saline)	ACTH A Gelatin	ACTH B Aqueous (0.5% phenol)	ACTH B Gelatin	Dose Totals	% Showing z.g. Depletion
.33	8/9[1]	7/9	7/8	6/8	28/34	82.5
.67	8/9	8/10	6/10	2/10	24/39	61.5
1.0	3/10	3/9	3/10	1/9	10/38	26.0
3.0	3/8	0/8	1/10	0/10	4/36	11.0
Treatment totals	22/36	18/36	17/38	9/37	66/147	
% showing z.g. depletion	61.2	50.0	44.8	24.3		
Controls 0.1 ml. saline	0/18					

[1] Numerator indicates number of animals showing lipid depletion in the adrenal zona glomerulosa. Denominator indicates number of animals examined. The difference between the denominator and 10 (or 20 in the case of the controls) indicates the number of animals dying at a time inconvenient for immediate post mortem examination. These were discarded.

Single, daily, subcutaneous injections beginning 24 hours post hypophysectomy.

tween the capsule and the lipid-rich cells of the zona glomerulosa which existed before pituitary ablation, acquired little or no lipid (Fig. 4).

Treated Series: In Table 1 are presented data on the depletion of sudanophilic components from the zona glomerulosa. The results are tabulated here without distinction as to time of death or sacrifice. Complete depletion of the glomerulosal lipids was established in the adrenals of 62 of 111 animals subjected to daily doses of ACTH through 1 unit (U. S. P.) per 100 gm. of body weight. The adrenal cortices of those animals failing to show depletion of glomerulosal lipid at doses below 1 unit were characterized by vacuolization, "exhaustion lipid," i.e. refractile luminescence of

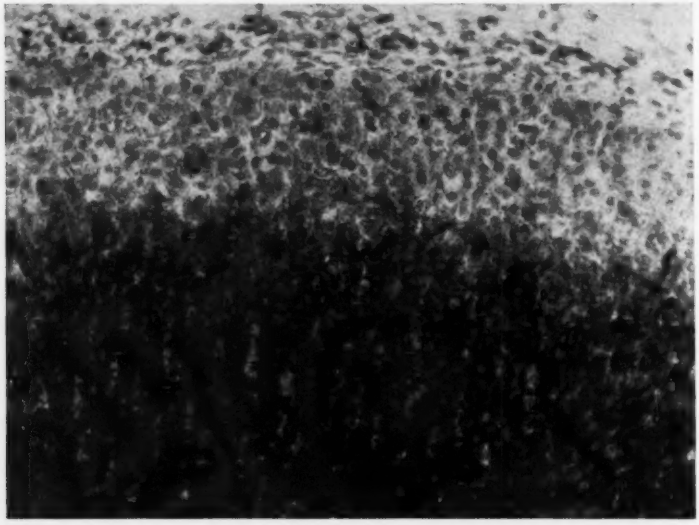

FIG. 5. Zona glomerulosa. $\frac{2}{3}$ USP unit ACTH in saline, subcutaneously, once daily, 4 days post-hypophysectomy. Zone cleared of all lipid. Zona fasciculata undisturbed (Sudan Orange, ×270)

lipid particles under ordinary illumination and cell necrosis (Figs. 10, 11 and 12).[2] Incipient, as well as complete lipid depletion of the zona glomerulosa was noted in the adrenals of animals dying two or three days after

[2] As used here, the term "exhaustion lipid" describes the histological appearance of the adrenal cortex following exposure of an animal to prolonged and severe stress culminated by death. In our experience such glands are traversed by maximally dilated capillaries coursing between cortical cells filled with lipid (coarse or clumped droplets). Tinctorially, the lipids stain a lemon-like hue (sudan orange) with many foci of refractile butter yellow-like clumps of lipid. (This is usually confined to the outer portions of the zona fasciculata). Individual cell membranes are sharply outlined. There is considerable vacuolization of cells, pyknosis, attended by multiple foci of necrosis. Lipid depletion from any particular zone may or may not be a feature of this condition.

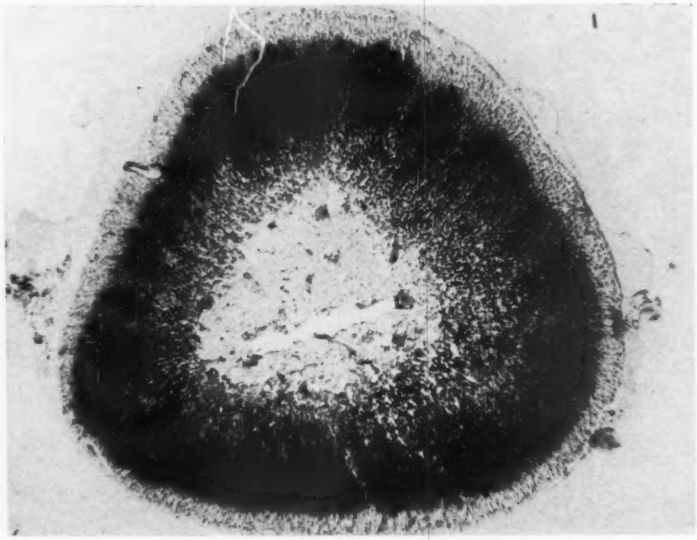

FIG. 6. Adrenal cortex. $\frac{1}{3}$ USP unit ACTH in saline, subcutaneously, once daily, 13 days post-hypophysectomy, zona glomerulosa depleted of lipid. Zona fasciculata not completely maintained. Lipid depletion of zona reticularis, extending into inner zona fasciculata. (Sudan Orange, ×25)

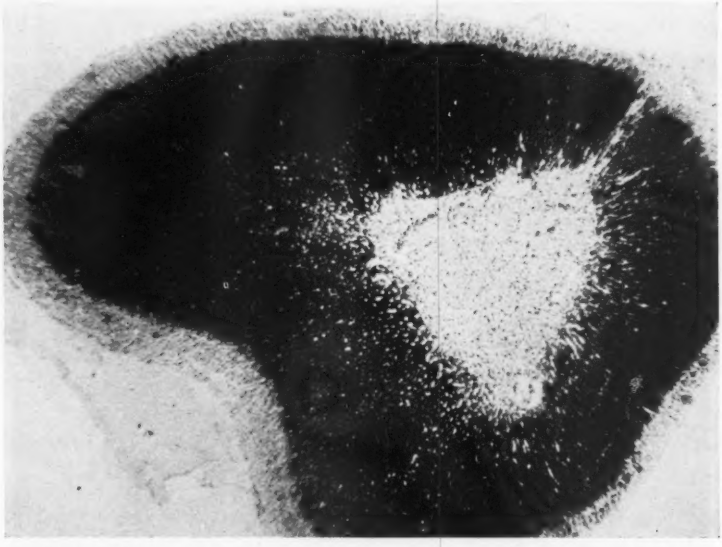

FIG. 7. Adrenal cortex. $\frac{2}{3}$ USP unit ACTH in saline, subcutaneously, 3 days post-hypophysectomy. Zona glomerulosa cleared of lipid. No evidence of atrophy. Some lipid depletion in zona reticularis along juxta-medullary border. (Sudan Orange, ×25)

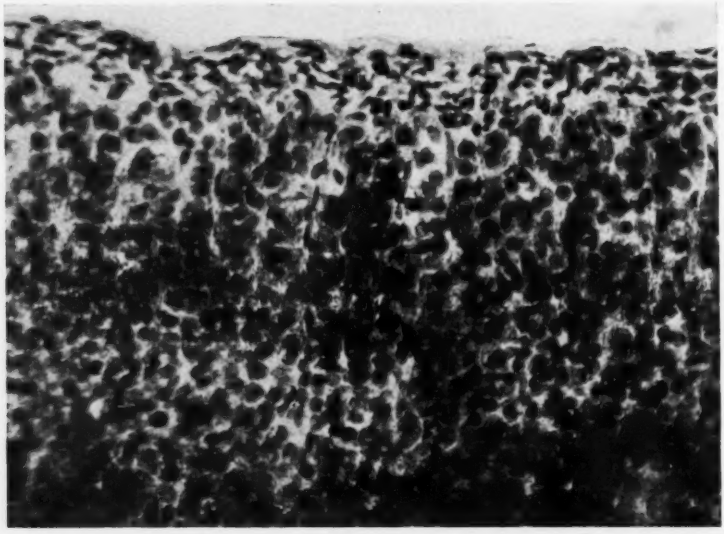

FIG. 8. Zona glomerulosa. $\frac{1}{8}$ U.S.P. unit ACTH in gelatin, subcutaneously, 3 days post-hypophysectomy, lipids show incipient depletion. (Sudan Orange, ×270)

hypophysectomy (i.e. following one or two injections of the hormonal material).

Following the third, fourth, and fifth injections the lipid of the zone had disappeared in 31 of 37 animals examined (Figs. 5, 6, 7, 8, and 9). The

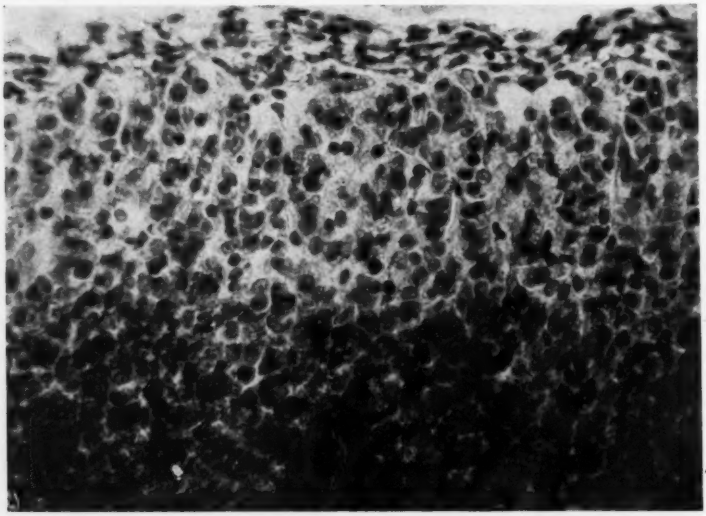

FIG. 9. Zona glomeruolsa. $\frac{1}{8}$ U.S.P. unit ACTH in gelatin, subcutaneously, 7 days post-hypophysectomy. Zona glomerulosa completely cleared of lipid. (Sudan Orange, ×270)

gelatin preparations produced a similar depletion of lipid in the zona glomerulosa (compare Figs. 4, 8 and 9). No evidence for reaccumulation and storage of lipid in the zone was detected.

When the dose of corticotropin was 3 units (U. S. P.) per 100 gm. body weight, few animals showed depletion of lipid in the zona glomerulosa (see Table 1). Instead, "exhaustion lipid" appeared in the cortex. Depletion of sudanophilic material occurred in the zona reticularis, inner fasciculata and portions of the outer fasciculata. The lipid present was highly refractile and clumped, the cells vesiculated or necrotic, the cortex itself

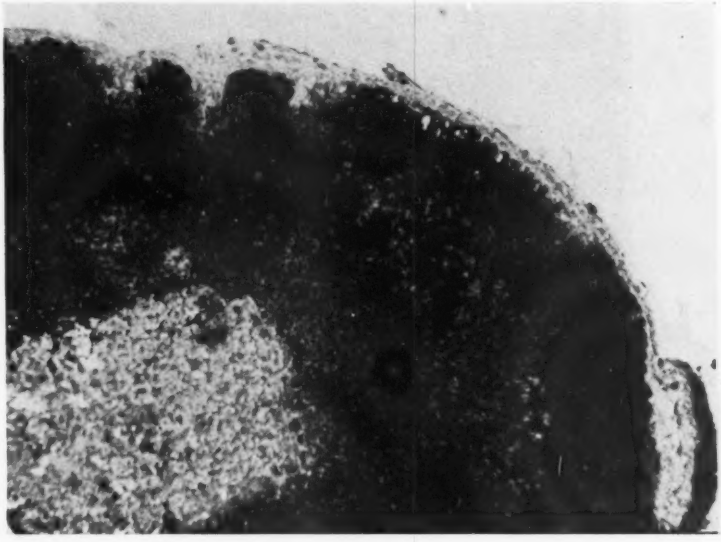

FIG. 10. Adrenal cortex. ⅓ U.S.P. unit ACTH in saline, subcutaneously, once daily 21 days post-hypophysectomy. Note lack of lipid depletion in zona glomerulosa. However, observe "exhaustion lipid" in zona fasciculata. (Sudan Orange, ×60)

hypertrophied and the reticular capillaries markedly dilated (Figs. 10, 11, and 12). As mentioned above, the same pattern was observed in the adrenals of animals failing to show depletion of the zona glomerulosa with doses of less than 1 unit of corticotropin. The animals were observed several times a day during the experimental period of 21 days and were removed for examination only when they became moribund or died during the time of observation. Extreme depletion of the sudanophilic elements in the zona glomerulosa was observed in 56 of 80 animals dying, or sacrificed in the moribund state, before day 21 among the ACTH-treated groups. In the animals surviving 21 days, 10 of 67 were found to have lipid-depleted zonae glomerulosa. Of these 67, 47 rats were in the groups receiving 1 or 3 units of ACTH daily. None of these 47 exhibited depletion of the

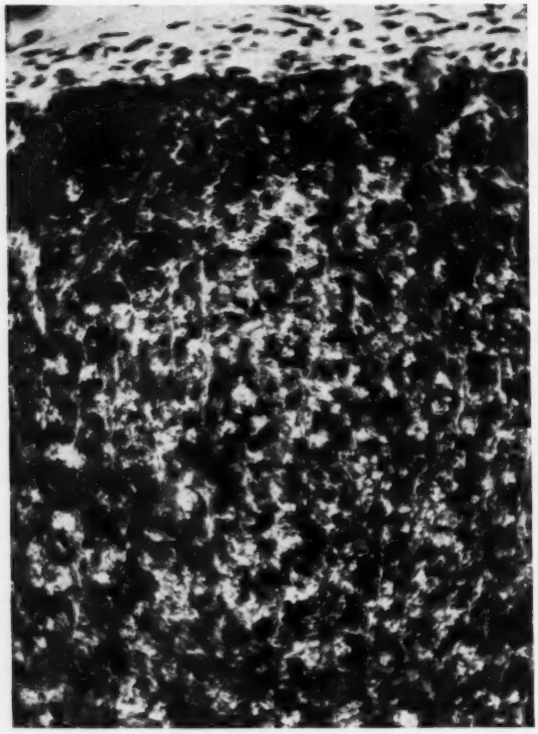

FIG. 11. Zona glomerulosa and zona fasciculata. ⅛ U.S.P. unit ACTH in gelatin, subcutaneously, once daily, 21 days post-hypophysectomy. Same as Fig. 10 (Sudan Orange, ×270)

TABLE 2. TIME OF DEATH AND STATUS OF LIPID COMPLEMENT IN ADRENAL ZONAE GLOMERULOSA OF HYPOPHYSECTOMIZED MALE SPRAGUE-DAWLEY RATS RECEIVING SINGLE, DAILY, SUBCUTANEOUS INJECTIONS OF ACTH PREPARATIONS AT VARIOUS DOSE LEVELS

Number of Injections Prior to Death	Controls Saline 0.1 ml.		ACTH "A"								ACTH "B"								Totals ACTH Treated	
			Aqueous				Gelatin				Aqueous				Gelatin					
			0.33–.67μ		1.0–3.0μ		0.33–.67μ		1.0–3.0μ		0.33–.67μ		1.0–3.0μ		0.33–.67μ		1.0–3.0μ			
	–[a]	+[b]	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+
2– 5		2	4	1	5	4	10		1	2	11		3	1	6	2	1	1	41	11
6–10		2	3	1		1	2	1			1	2	1	1	2	1		1	9	8
11–15		2	1		1		2		1	1						1		1	5	3
16–20		1							1			2							1	2
21[c]		11	8			7	1	3		11	1	1		14		6		15	10	57
Totals	0	18	16	2	6	12	15	4	3	14	13	5	4	16	8	10	1	18	66	81
Totals through 20 days	0	7	8	2	6	5	14	1	3	3	12	4	4	2	8	4	1	3	56	24

Daily dosage in U. S. P. units per 100 grams body weight
a) – indicates extreme depletion of sudanophilic material in adrenal zona glomerulosa.
b) + indicates presence of sudanophilic material in adrenal zona glomerulosa.
c) Animals surviving the experimental period were sacrificed 24 hours after the 21st injection.

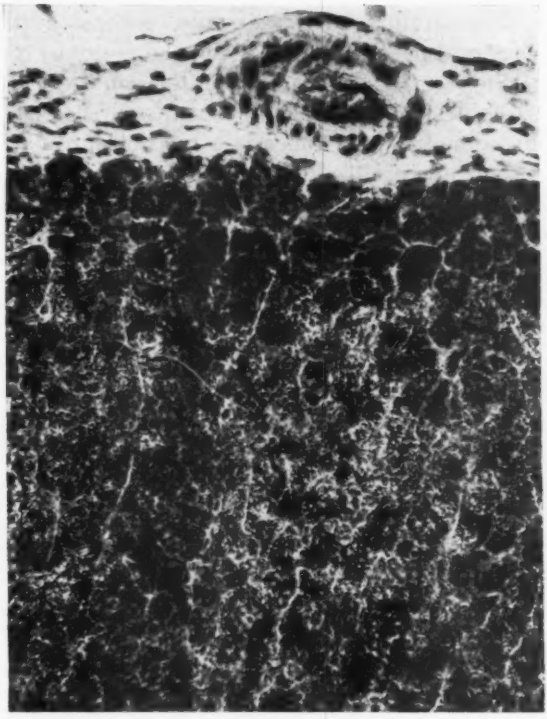

FIG. 12. Zona glomerulosa and zona fasciculata. 3 U.S.P. units in gelatin, subcutaneously, once daily, 21 days post-hypophysectomy. Severe exhaustion. (Sudan Orange, ×270)

sudanophilic elements in this zone. The adrenals of 7 of 9 animals dying before day 21 in the control groups were examined and found to have apparently normal complements of lipid in the zonae glomerulosa. Table 2 summarizes these results.

DISCUSSION

The hormonal preparations used in these studies were of pituitary origin. The results support the concept of pituitary control, in part at least, of the sudanophilic elements in the adrenal zona glomerulosa of the rat. The physiological significance of such evidence is obscure. That mobility of the zona glomerulosa lipid can be associated with abnormalities of electrolyte intake has been demonstrated by Deane, Shaw and Greep (1948). A prolonged sodium-deficient diet (38–70 days) produced almost complete depletion of the lipid in this zone in intact and hypophysectomized rats. Similarly, the injection of KCl solutions caused acute depletion in the glomerulosa of intact rats. The time interval in which such depletion was

observed was different for the experiments of these authors involving sodium deficiency and potassium excess and the present experiments with corticotropin. However, an apparently identical end result, i.e. depletion of an otherwise stable complement of lipid in the adrenal zona glomerulosa of the hypophysectomized rat, suggests a relationship involving the hypophysis, that zone, and electrolyte balance.

The studies of Ingle *et al.* (1946, 1947) and Bergner and Deane (1948) do not provide evidence for such a relationship. These authors report that ACTH is without effect on the electrolyte balance of the rat. Our observations show that some ACTH preparations have a marked effect on the lipids of the adrenal zona glomerulosa in the rat. If it is assumed that the hormone preparations used in the earlier work are comparable in composition to those used in our investigations, an assumption not without risk, it follows that the depletion of sudanophilic material from the zona glomerulosa, induced by material of pituitary origin, is not necessarily related to electrolyte balance in the rat.

The assumption of comparable composition of the corticotropic preparations is, however, probably unwarranted. Although all pituitary fractions marketed as ACTH are characterized by their capacity to deplete adrenal ascorbic acid in the hypophysectomized rat, not all such materials in our experiments have induced lipid depletion in the zona glomerulosa (Wexler *et al.*, 1955). Only preparations exhibiting both activities were used in the present work. Clearly, further investigation requires the study of electrolyte balance in the rat employing pituitary fractions known to induce lipid depletion in the zona glomerulosa of that animal.

The histological finding of an inverse relationship between corticotropin dose level and the incidence of lipid depletion in the adrenal zona glomerulosa is subject to more than one interpretation. Elaboration on this point involves speculation which, until further investigation is carried out, would serve no useful purpose.

A marked difference in the proportion of animals showing depletion of the sudanophilic components of the zona glomerulosa in the gelatin ACTH-treated groups of brand B is apparent when comparison is made with all other groups. It would appear unlikely that this difference is attributable to the suspension of the hormone in the gelatin menstruum since no pronounced difference in these proportions was noted between the menstrua of the brand A-treated groups. A more plausible explanation would suggest a difference in composition in the particular lot of hormonal material used in the gelatin preparation of brand B.

The incidence of depletion of the sudanophilic components in the adrenal zonae glomerulosa of animals dying, or sacrificed in the moribund state, before day 21 was, on a percentage basis, more than four times that of the animals surviving the entire experimental period. The proportion of deaths

in the groups receiving 1.0–3.0 units (U. S. P.), in which 19% of the animals showed glomerulosal depletion, did not differ significantly from that in the saline-treated controls. In contrast, the groups receiving less than 1 unit of ACTH as a daily dose, and in which 71% of the animals showed glomerulosal depletion, had a greater proportion of deaths before day 21 than the controls, none of which exhibited such depletion. The Chi-square test applied to these proportions indicated a probability between 0.05 and 0.02. The same test applied to the proportion of deaths in the groups receiving less than 1 unit and that in the groups receiving a daily dose of 1 unit or more gave a probability value less than 0.001.

It is readily apparent that many factors may be responsible for the death of an hypophysectomized animal, particularly in the immediate postoperative period. However, the identity of conditions for all animals, including a single operator for the surgical procedure, suggests that there should have been no difference in the proportions of the animals surviving the experimental period in the various groups. That significant differences in these proportions were observed implies a negative association between the capacity to survive the experimental conditions and daily dose levels of ACTH below one unit. On the other hand a positive association between the capacity to survive and daily dose levels of one unit or more was not indicated when reference was made to the saline-treated controls.

SUMMARY

The effects of prolonged administration of ACTH preparations, in gelatin and in aqueous solution, on the adrenal zona glomerulosa of the hypophysectomized, male Sprague-Dawley rat have been studied by histological techniques. The results demonstrate an action by material of pituitary origin on the zona glomerulosa of the rat adrenal cortex. At doses below one unit (U. S. P.) the adrenal lipid pattern was in sharp contrast to those receiving one unit or more. Complete, or almost complete, depletion of the sudanophilic elements of the zona glomerulosa was observed at the lower dose ranges in 52 of 73 rats. Above one unit few animals showed depletion of glomerulosal lipid. Instead, depletion of sudanophilic material occurred in the zona reticularis, inner zona fasciculata, and portions of the outer fasciculata. In contrast to the adrenals from animals receiving less than one unit, the lipid present was highly refractile and clumped, the cells vesiculated or necrotic, the cortex itself hypertrophied and the reticular capillaries markedly dilated.

Significant differences were observed in the proportions of animals surviving the experimental conditions in the various groups. Those receiving less than one unit of ACTH daily showed a greater proportion of deaths than the saline-treated controls. The groups injected with one unit or more daily did not differ significantly from the controls in this respect.

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ELECTRON MICROSCOPIC OBSERVATIONS ON THE NORMAL AND DENERVATED ADRENAL MEDULLA OF THE RAT[1]

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MULON (1905) first observed that adrenal medullary cells when fixed with osmic acid exhibit intracellular osmiophile granules. Assuming these bodies to be comparable with the chromaffin granules of Hultgen and Anderson (1899) and Kohn (1902), he claimed that the osmic acid staining reaction was a specific histochemical test for adrenalin. Bennett (1941) disputed such an absolute specificity on the grounds that, besides adrenaline itself, other reducing substances (many of them polyphenol precursors of adrenaline) were blackened by osmic acid.

Since osmic acid has recently proved effective as a tissue fixative for electron microscopic study (Porter, Claude and Fullam 1945: Palade 1952: Dalton 1955), it seemed especially relevant to examine the adrenal medulla by this method.

MATERIAL AND METHODS

The Normal Medulla. During the electron microscopic study of the rat adrenal cortex (Lever (a) in press) the medulla was concurrently examined in some 25 animals. In addition 12 more normal glands were obtained as controls in the denervation experiments described below.

The Medulla after Cold Exposure. Six rats (in wet cages) were exposed to a temperature range of 3–5° C. for two hours, after which their adrenals' were removed for examination.

The Denervated Medulla. Left-sided adrenals were denervated by incising the loose connective tissue of the posterior abdominal wall along a line starting lateral to the gland, arching above it, and then extending downward and medial to it as far as the middle adrenal artery (Vogt 1945). The destruction of the superior adrenal artery during this operation resulted in a localized infarction extending from the subcapsular cortex to the cortico-medullary junction. Animals were sacrificed at 24 hours (4), 48 hours (4) and 72 hours (4), after denervation, and both adrenals removed for examination (right-sided adrenals being the normal controls).

In all instances animals were killed by a blow on the head and the chest wall was immediately opened; 5 cc of Dalton's (1955) osmic-dichromate fixative were injected

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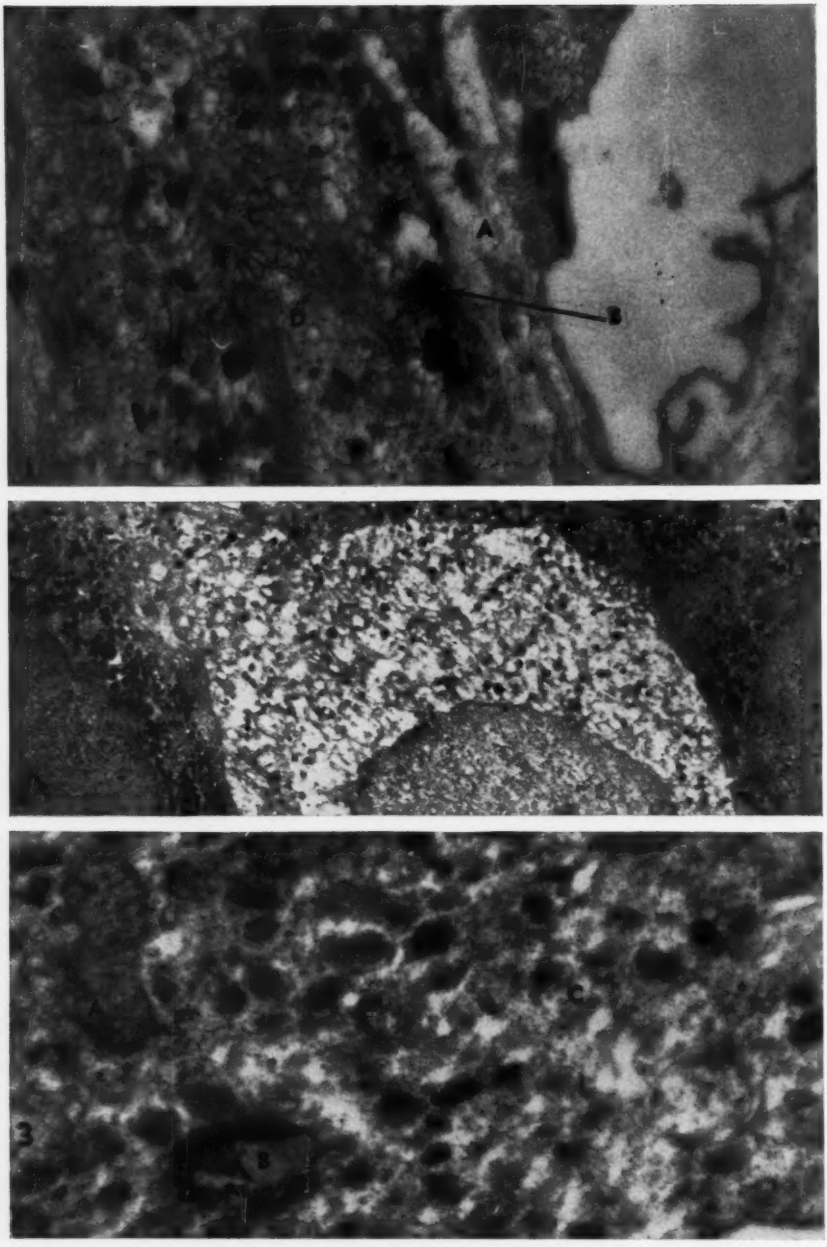
[2] On leave of absence from the Department of Anatomy, Cambridge University, Cambridge, England.



EXPLANATION OF PLATE 1

FIG. 1. From the normal rat medulla. Extending up to the right is a blood sinusoid with a convoluted endothelial lining. Within the subendothelial space (A) are imbri-

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into the thoracic aorta. On immediate sectioning of the adrenals with a keen razor, an already blackened medulla was clearly outlined. Fixation of small (1 mm.) pieces of tissue was continued for a further 40 mins. by immersion in Dalton's fluid, after which dehydration, methacrylate embedding, and microtomy was carried out as previously reported (Lever (a) in press). Sections in the order of 150–200 Å were examined by an RCA electron microscope (E.M.U. 2E).

OBSERVATIONS

A. *The Normal Rat Medulla*

By low power observation with the electron microscope and by phase microscopy, the histological arrangement of the medulla conforms to conventional descriptions by light microscopy (Rogoff 1932). Thus the cells are rounded or polygonal in shape and arranged in groups or columns which anastomose in irregular fashion: between the cell columns are numerous capillary sinusoids, reticular fibers, and both myelinated and unmyelinated nerve fibers. In Bennett's (1941) excellent treatise on the cat medulla ample space is devoted to a critique of earlier investigations of medullary cytology. Cramer (1928) recognized two sorts of parenchymal cell in the mouse adrenal medulla; a dark form containing fine osmiophile secretory granules and a lighter variety (according to him, more numerous in the stimulated medulla) from which he claimed the granules had been, or were being, discharged. Hion (1927), using formol-dichromate-fixed rat adrenals stained by the Altmann-Kull method, described four categories of medullary cell; a, b, c and d: "a" and "d" cells were agranular and supposedly non-secreting while "b" and "c" cells, because of a content of fuchsinophile granules were, according to him, actively secreting forms.

Because of the obvious difficulty, by electron microscopy, of assessing the tissue as a whole and because of the complexity of earlier cytological observations by light microscopy, it was felt that in the interests of clarity, the present account should deal with two main cell categories as seen with the electron microscope: cells, "light," "dark" and intermediate, as judged by their overall electron density, have been observed in all medullae in this study.

The cellular-vascular relationship. Between the parenchymal cell membrane and the endothelial lining of the regional blood sinusoid there is a

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cated portions of endothelium and a semi-opaque material. The parenchymal cell plasma membrane is of doubtful integrity in some places (B). Within the medullary cell are seen deeply osmiophile adrenaline granules, obviously granular walled ergastoplasm (C) and sacs of equivocal appearance (D). ×35,000.

FIG. 2. Dark and light cells depicted in the normal medulla. ×9000.

FIG. 3. Normal medullary cell cytoplasm: the mitochondrion (A) exhibits a laminar internal structure: (B) is probably a pigment-containing mitochondrion: at (C) and elsewhere a fine-walled sac surrounds a collection of microgranules which are probably precursor components of an adrenaline granule (see Fig. 12) ×27,000.

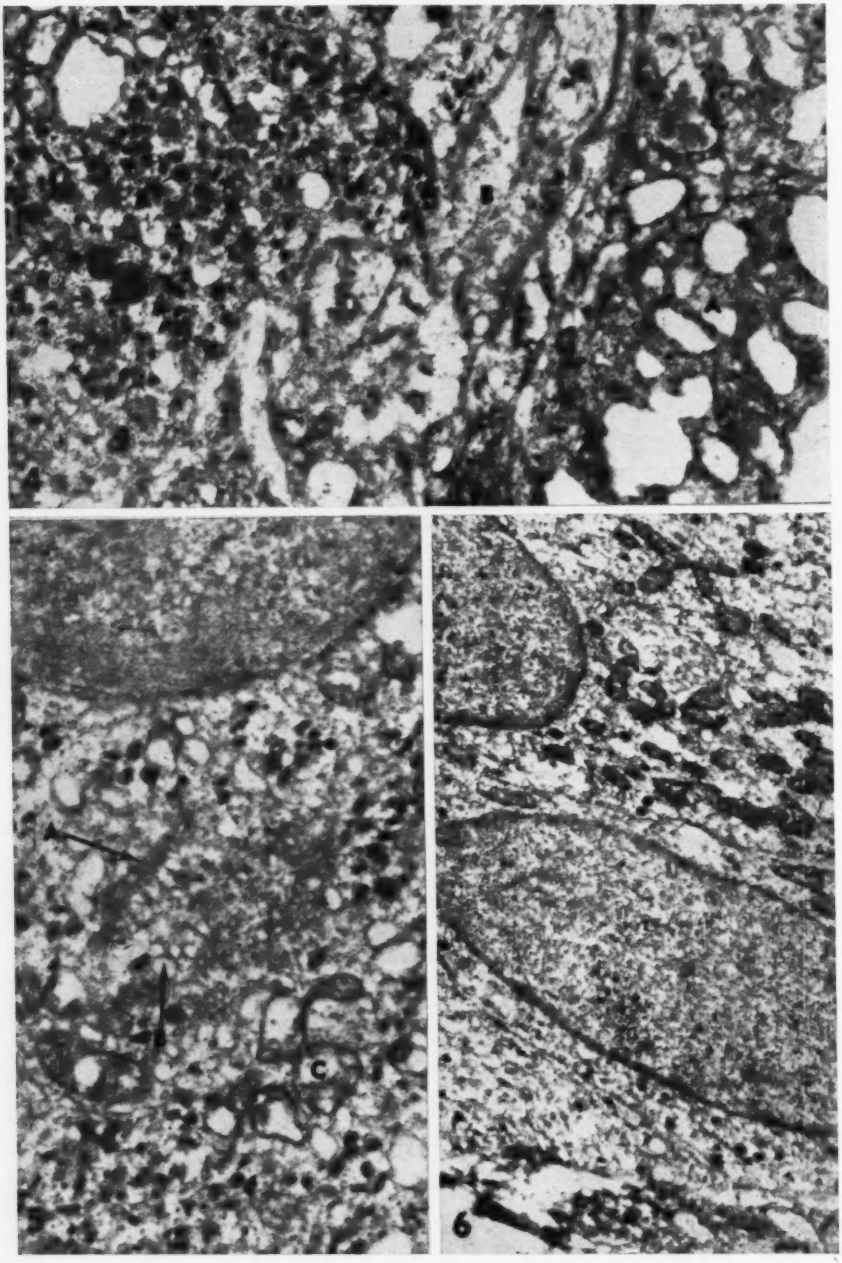

PLATE 2

space (Bennett 1941) in which occasional wisps of collagen and sometimes (particularly in some regions of the stimulated medulla) a semiopaque amorphous substance can be seen (Fig. 1). As in the adrenal cortex and corpus luteum, where similar subendothelial intervals have already been described (Lever, a and b, in press), that in the medulla communicates with interparenchymal-cell spaces and from it short extensions may invaginate the medullary cell plasma membrane though not to the same degree as seen in the adrenal cortex. Osmiophile "adrenaline" granules as such have not been observed either in the subendothelial space or the sinusoid lumen: this does not substantiate Cramer's (1928) claim that medullary secretion was associated with an actual discharge of these bodies *per se* (v.i.).

The Medullary parenchymal cells. As already indicated, dark light and intermediate cell forms make up the medullary parenchyma (Fig. 2). To explain the term *dark* cell, it must be added that this is used in a relative, not an absolute sense, the implication being that such a cell is *darker*, i.e. generally more electron opaque than the light cells.

As in many other cells in osmic-fixed preparations the background cytoplasm of the medullary cell is semiopaque, and homogeneous at the preent limits of electron microscopic resolution. This applies equally to the finely granular nucleoplasm which is contained by a bilaminar nuclear membrane.

The Golgi apparatus is usually in the form of a collection of thin-walled sacs and or a sheaf of several exceedingly fine-walled microtubules. A compact medullary Golgi complex is commonly seen in a paranuclear position (Fig. 5) but this does not imply a strict localization of the organelle portions of which are frequently more widely disseminated through the cytoplasm.

Some ergastoplasmic material is easily recognizable as fine sacs or tubules with a definitive linear inner wall and an irregular granular outer covering

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EXPLANATION OF PLATE 2

FIG. 4. Forty-eight hours after denervation of the medulla: many of the sinusoid lining endothelia are crumpled or imbricated (A): there is an absence of detail in nerve tissue (B): within the medullary cell most of the adrenaline bodies are irregularly shaped and have halos of less dense material around them: the cytoplasm is variably vacuolated. ×18000

FIG. 5. Part of a light cell from normal medulla. In center field is a Golgi complex with fascicle of microtubules (A), polyhedral sacs (B). Note that all the visible mitochondria are variably vacuolated (C). Distinction between vacuolated mitochondria and the Golgi sacs (B) is highly equivocal. ×21000.

FIG. 6. Medullary cells from a rat after low temperature exposure. Note, the cytoplasm though much depleted of adrenaline granules, is not absolutely agranular. The mitochondria (top right) stand out in sharp relief in an otherwise pale background. ×13500.

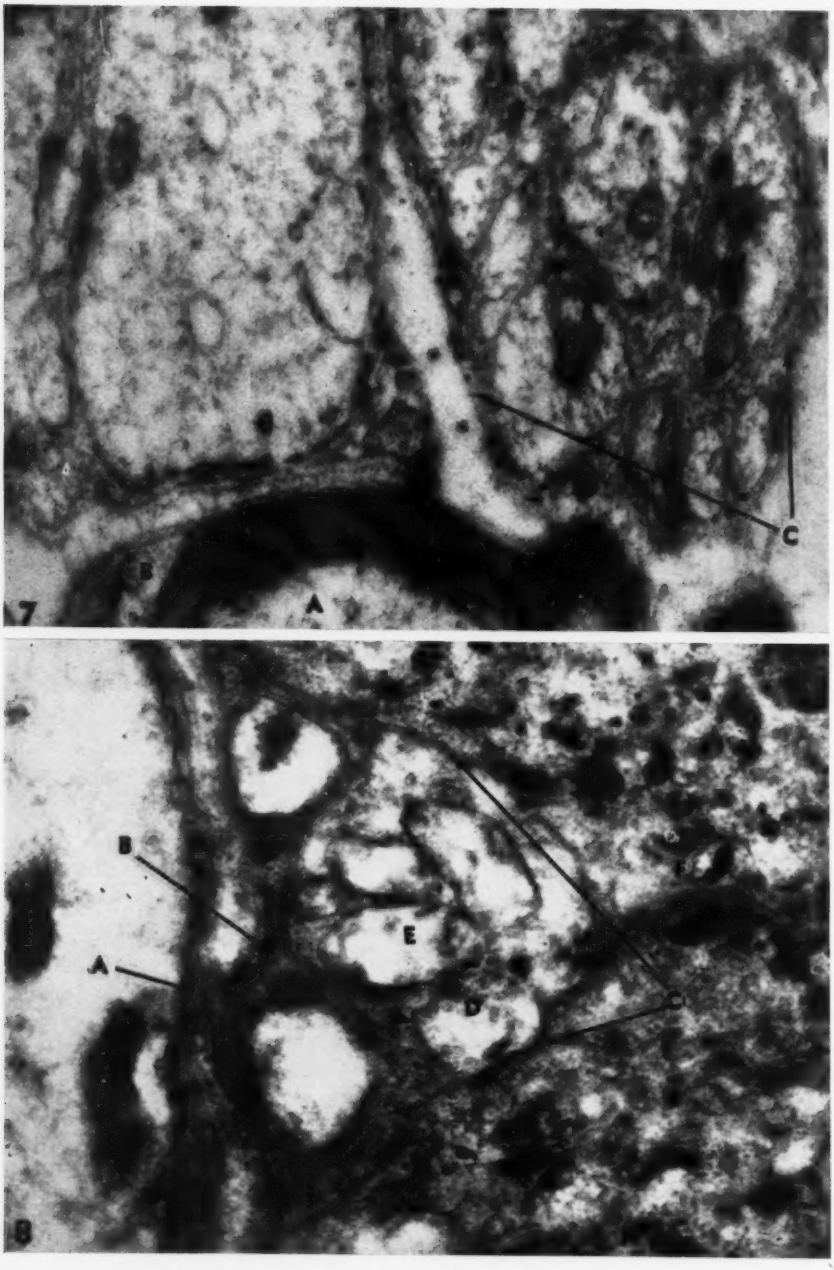

PLATE 3

(Fig. 1). There are in addition numbers of cytoplasmic saccules which, because of their small size, defy accurate description, but which nevertheless are probably ergastoplasmic in nature (Fig. 1). Osmiophile secretory granules have been observed without exception in every medullary cell examined in this study though they are found in largest number in the dark cells. By light microscopy of the cat medulla, Bennett (1941) observed many agranular cells; but in many instances the cytoplasm of these, being rich in reducing substances, was strongly osmiophile: he regarded such appearances as "presecretory" since, according to him, actively secreting cells always contained granules. The appearance of secretory granules in electron micrographs is varied: they range in form from loose collections of component microgranules (Figs. 12 and 3) to discrete sharply defined homogeneously and intensely osmiophile bodies of variable size (Figs. 1, 2, and 8). In a large number of instances they appear to be invested by a delicate membrane or sac (Figs. 12, 8 and 3) and distinction between such sacs and those already described as ergastoplasmic or even Golgi-form is highly equivocal. On occasions a sharply defined granule within such a sac may be surrounded by a halo of less dense material. It is as difficult to prove as it is to absolutely deny the possibility that the osmiophile secretory granules appear within *ergastoplasmic sacs;* as is the case with zymogen granules in the pancreas (Weiss 1953) and the colloid bodies of the thyroid (Dempsey and Peterson 1955).

A duplicity of the mitochondrial outer membrane is only occasionally obvious: for the most part this appears as a single line. On the other hand the bilaminar nature of the mitochondrial cristae (when these constitute the membranous internum) is usually apparent (Fig. 3). There is considerable variation in the internal appearance of the mitochondria throughout a medullary field, but within individual cells it is usually remarkably constant. In general, in the normal gland, dark cell mitochondria show a compact laminar internum, while in lighter cell forms they may be irregularly vacuolated with a loose filamentous or even saccular internum (Figs. 5 and 12). In the stimulated adrenal however, many of the cells, though

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EXPLANATION OF PLATE 3

FIG. 7. Nerve fibres within the adrenal medulla. (A) is a single axon invested by ringed myelin, external to which is Schwann cell cytoplasm (B): compare with (A) in figure 9. Several unmyelinated axons are invested by Schwann-cell cytoplasm (C): again compare with figure 9. ×20000.

FIG. 8. Synaptic region in rat medulla. To the left is an unmyelinated axon covered by Schwann cell (A). Schwann cell cytoplasm and membranes are similarly seen at (B). In center field is an appearance which may be a nerve termination or bouton on the parenchymal cell (right). At (C) note that no Schwann cytoplasm separates the parenchymal and axonal plasma membranes. Within the bouton are numerous microvesicles (D) and some vacuolated mitochondria (E). Note the delicate membranous investment of adrenaline granule at (F). ×22500.

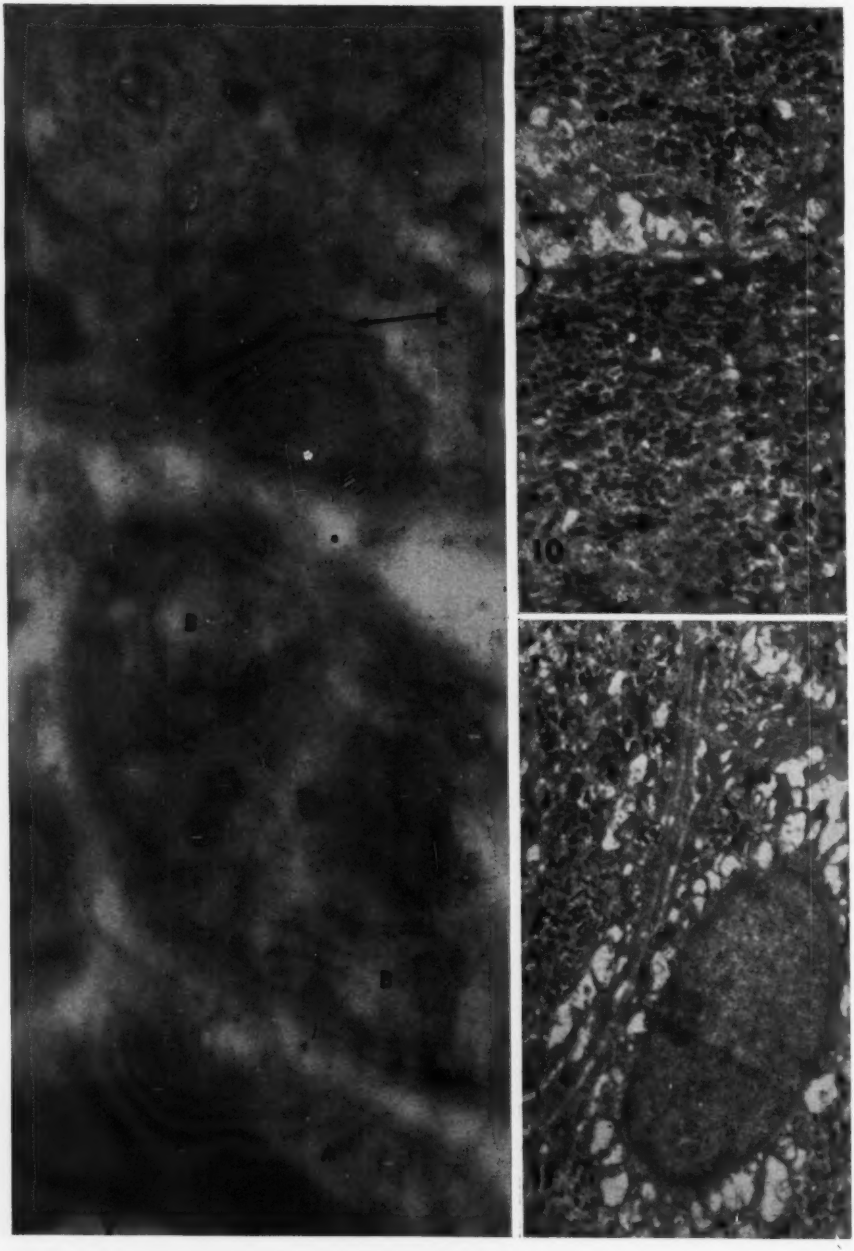

PLATE 4

partly denuded of secretory granules and with a paucity of osmiophile background substance (hence light in appearance), do nevertheless contain mitochondria of compact internal structure. An advanced degree of mitochondrial vacuolation is seen in the light cell in figure 5. Many of the thin walled sacs portrayed are probably an end result of this process of mitochondrial vacuolation and destruction, the significance of which is obscure. Although a three dimensional concept of their shape is not feasible at the present, the medullary mitochondria are predominantly oval or elliptical in section.

Within many medullary cells an occasional mitochondrion is found to contain irregular aggregations of a deeply opaque substance (Fig. 3). The sharpness in outline and granular appearance of this material suggests a pigmentous rather than a lipid consistency. Hess (1955) has observed similarly loaded mitochondria within posterior root ganglion cells.

B. *The Stimulated Medulla*

There is a noticeable reduction in the amount of secretory granular substance and to a lesser extent of osmiophile background material in many, though not all, of the medullary cells following cold subjection of the animal. As Bennett (1941) originally observed by light microscopy, in the cell thus depleted (Fig. 6), the mitochondria usually stand out prominently against a pale background: on occasions they may have a loose vacuolated internum, but in many electron micrographs this is compact and laminar in outline. Other cytological features are as described above for the normal medulla. Whether or not such a depleted cell should be considered as synonymous or comparable with the *light* cell (already mentioned as a normally occurring medullary constituent), is a question best discussed at the end of the paper. However it is well to reiterate, that no cell was ever found to be completely agranular and that not by any means

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EXPLANATION OF PLATE 4

FIG. 9. Nerve fibres and a synaptic region, 24 hours after denervation. Myelin rings investing an axon are externally covered by Schwann-cell cytoplasm (A): this covering is more obvious than in the normal myelinated fibre (Fig. 7). Several shrunken unmyelinated nerves (B) are invested by a Schwann cell; mesaxons may be whorled and complex (C). Towards the top of the print a probable bouton is seen to contain opaque compact mitochondria (D) but no obvious microvesicles. At (E) the plasma membranes of bouton and parenchymal cell are in juxta-position. ×17000.

FIG. 10. Twenty-four hours after denervation many medullary cells are more electron dense than normal with a crowding together of adrenaline granules, many of which are less opaque than normal. ×13500.

FIG. 11. Forty-eight hours after denervation many medullary cells are markedly shrunken: their cytoplasm lacks detail and is frequently fenestrated. There is a noticeable absence of microgranular secretory forms as in figures 3 and 12 and the adrenaline granules are irregular and reduced in average size over the normal (also see Fig. 13). ×12000.

all of the medullary cells are obviously depleted of secretory granules on stimulation.

In his study of the effects of a lowered body temperature on the chromaffin reaction in the rat medulla, Vincent (1925) noted a patchy distribution of the cell groups in which the reaction was clearly of reduced intensity. According to him, in many adjacent medullary cells, chromaffin staining was unaffected by such stimulation. An essential patchiness in the distribution of cells depleted of granules, has been observed in the present study. In contrast, Bennett (1941) described an intensification of the chromaffin reaction and an increase in the number of "secretory" granule-containing cells in the cat medulla after splanchnic nerve stimulation (for one minute).

C. *Nerves in the Adrenal Medulla*

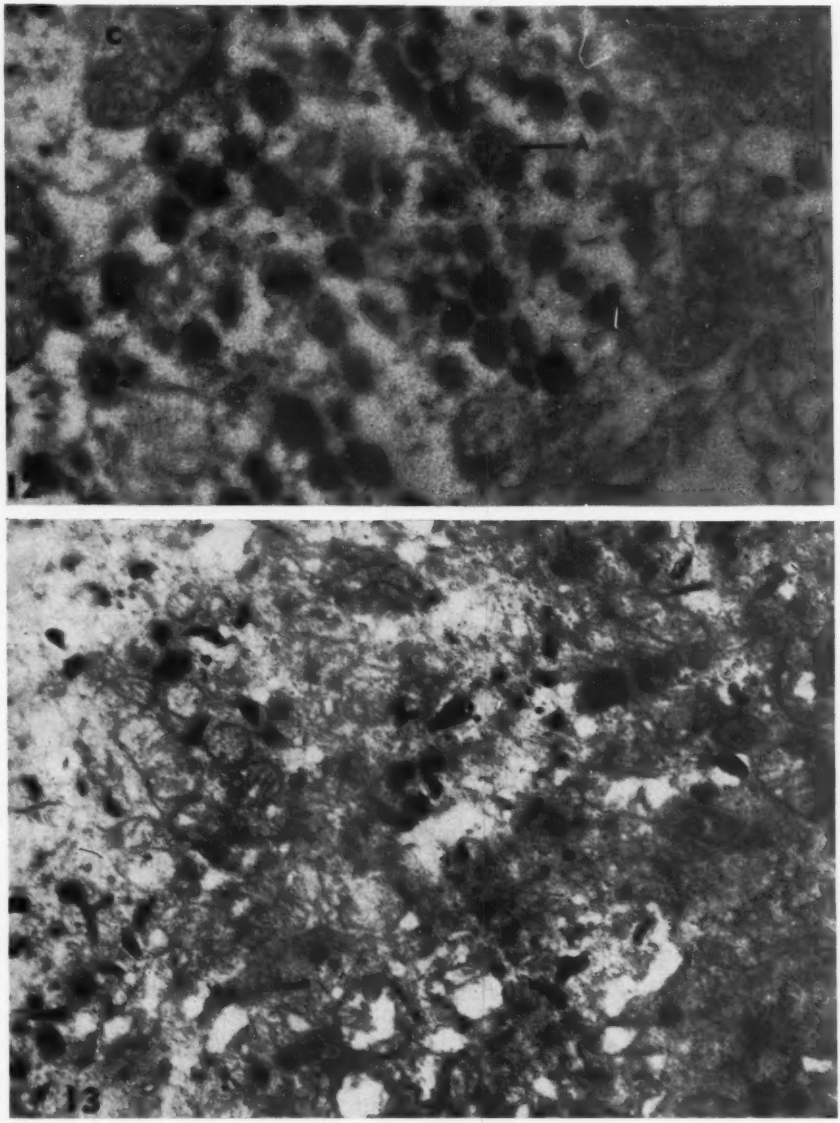
Bundles of both myelinated and unmyelinated nerve fibers lie in the loose vascular connective tissue between the parenchymal cell columns. Myelinated fibers are more frequently seen near the periphery of the medulla and in electron micrographs, apparently consist of a single axon enclosed by a sheath of myelin rings (Fig. 7) as described by Hess and Lansing (1953). On the other hand a single Schwann cell is seen to invest several unmyelinated axons (Fig. 7).

Wherever other appearances strongly suggested a nerve termination on a medullary cell, axoplasm and cell cytoplasm were only separated by their respective plasma membranes (Fig. 8). Elsewhere, whenever an axon was opposed to a parenchymal cell, there was always the additional interposition of a Schwann cell cytoplasm and its plasma membrane. Besides this feature, these synaptic regions were characterized by a concentration of several parenchymal cell mitochondria in the immediate vicinity, and by the presence of numerous micro-vesicles within the axoplasm of the bouton. Bennett (1941) in the cat adrenal, postulated that the medullary nerves were distributed along the course of the arterial capillaries and hence terminated on the parenchymal cell at or near its ("nutrient") capillary pole. In the electron microscopic study of the rat adrenal, although nerve fibers are abundant, unequivocal identification of synapses is difficult: however in some six examples of nerve termination it would seem that Bennett's polarity concept, in this respect, is true also for the rat medulla.

D. *The Denervated Medulla*

In his denervation experiments on the cat medulla, Young (1939) observed parenchymal cell degeneration in from 5–8 days after operation. In the present series what might be construed as suggestive evidence of degeneration was discernible before the third day and at 9 days all cytological detail was absent.

24 Hours After Denervation. (a) The subendothelial spaces and the intercellular intervals between the medullary cells are accentuated, suggesting



EXPLANATION OF PLATE 5

FIG. 12. From a normal medullary cell. Varying appearances of the adrenaline secretory bodies may represent a series of transitions from a stage (A) which suggests a microgranular composition of these, through intermediates (B)t o the fully formed adrenaline granule. Note the saccular investment of (A). Mitochondria (C) have a filamentous or saccular internal structure. ×27000

FIG. 13. Three days after medullary denervation the adrenaline granules are highly polymorphic and many appear to be patchily eroded. There is a noticeable absence of microgranular secretory forms as in figure 12(A). ×17000.

a parenchymal tissue shrinkage. (b) Like the normal gland there is still an apparently random distribution of light and dark cells but following denervation many of the latter appear more electron dense than normal (Fig. 10). The cause of this increased opacity is twofold; a crowding together of secretory granules as a result of cell shrinkage; and a greater electron density of the background cytoplasm which could either be absolute or merely represent compactness of this material in a shrunken cell. (c) It is a definite impression that a number of the secretory granules in both light and dark cells are themselves less osmiophile; many of them showing pale central or eccentric areas. There is a noticeable reduction in the number of secretory bodies (granules) exhibiting the fine microgranular consistency (i.e., a microgranular consistency of a secretory *granule*) frequently observed in the normal medulla. (d) The basic cytological architecture of the medullary cell remains the same as normal and the mitochondria exhibit their normally occurring variations (vide supra). (e) In both myelinated and unmyelinated nerves the neurofibrils are not as clearly defined as in the normal. (f) There is a shrinkage in size of axon diameter and ensheathing the unmyelinated nerves the Schwann cell cytoplasm appears much more abundant than in the normal gland, with well marked infoldings of its plasma membrane providing a whorled and complex mesentery around the attenuated axons (Fig. 9). Schwann cells, usually stretched as a thin investment over the axons, are much more condensed when the latter are degenerating and so are a more prominent cytological feature under these conditions of denervation. (g) At the synaptic region, neither the microvesicles (described in the normal) nor the neurofibrils are clearly present in the axoplasm (Fig. 9).

The Denervated Medulla after 48 hours and beyond. There is an accentuation with time of many of the above mentioned cytological changes. Of particular note is the extreme irregularity of form of the secretory granules seen in many (though not all) of the medullary cells. In some cells 48 hours after denervation, the granules may appear jagged or in thin rod or comma shapes and many are noticeably surrounded by halos of less opaque material (Figs. 4 and 13). At 48 hours the ensemble of the granule plus its halo, is usually still confined by a delicate membrane identical with that described in the normal gland, but later than three days after denervation such a saccular outline is often missing, when the now smaller, more irregular, granules apparently lie free within the cytoplasm (Fig. 13).

A significant corollary to the disappearance of the membranous investments of the secretory granules is a contemporaneous absence of frankly ergastoplasmic organelles. The number of vacuolated mitochondria throughout the tissue increases with time after about the 3rd day following denervation and in fact the numerous cytoplasmic fenestra in some cells at this period are probably the sequelae of mitochondrial vacuolation (Fig. 11). Cytological detail of nervous tissue becomes increasingly obscure with

time after denervation. Lastly, owing presumably to medullary cell shrinkage, the endothelial lining of the larger (venous) sinusoids become much folded and imbricated after the third day following denervation so that it presents an appearance as in Figure 4. Just as there is an admixture of light, dark and intermediate cell forms comprising the *normal* medullary parenchyma, this heterogeneity remains a feature of the denervated gland right up to the complete degeneration of the tissue.

DISCUSSION

As far as can be ascertained no previous information exists on the site of formation and fine structure of the medullary secretory granules. There is considerable normal variation in the size and form of these bodies and it is certain that many of the smaller granules seen in electron micrographs would be well beyond the limit of resolution for light microscopy. Thus in the earlier studies (by light microscopy) many cells must have been described as agranular or possessing few granules, while it would seem (by electron microscopy) that truly agranular cells are at any rate not a feature of the normal rat medulla. This fact alone makes comparison with earlier works highly confusing on many points.

As already reported, the appearance of the secretory granules in the normal medullary cell ranges from a collection (loose or otherwise) of micro-granules, to densely osmiophile spherical or near spherical bodies of variable size: between these two extremes there are intermediate arrangements. All of these granular forms can be seen on occasions to have a fine membranous investment; a finding which supports a conception that they are inter-related and may represent different stages in adrenaline manufacture and release. This view is supported by the following argument. After denervation of the medulla, micro-granular aggregate secretory forms are rare, and the secretory granules tend to be irregular in shape, many show patchy translucencies and a large number appear to be surrounded by halos of less dense material (Fig. 4): conversely these last two granular forms, which strongly suggest a granule dissolution, are present in the gland of the normal resting animal though not to such an obvious extent. It is an established physiological fact that following denervation not only is the output of adrenaline from the medulla reduced, but also is its manufacture. If therefore in such a denervated medulla there is a noticeable absence of the microgranular composite secretory forms which are abundant in the normal, then such forms may be representative of granule formation and so indirectly be a detectable indication of adrenaline synthesis. If this be so then the halo forms and irregularly translucent granules could be an expression of adrenaline release: the very fact that adrenaline release is slow in the denervated medulla may account for the preponderance in it of these latter types of secretory body. From all these various arguments, this work lends support to Bennett's (1941) contention

that the osmiophile, chromaffin ("fuscogenic") granules represent actual concentrations of adrenaline.

The exact nature of the saccular investments of the secretory granules is indefinite, although an argument for this being ergastoplasmic can be made. In some locations they simulate in size and appearance other saccular bodies which have an outer granular covering over a definitive inner membrane and which therefore might be identified as ergastoplasm (Weiss 1953). Furthermore in medullary cells more than three days after denervation, neither obvious ergastoplasmic nor peri-granular sacs can be discerned (Fig. 11).

Perhaps on the strength of a comparatively limited investigation and because of the many unresolved queries, it would be unwise to attempt a comprehensive cell classification for the rat medulla as did Hion (1927). However it would appear that cells described as *dark* in this study are primarily dark because of a high granule content, while the *light* cell is comparatively lucid primarily because of a lower granule content. Assuming a granule depletion to be an accompaniment of adrenaline output, then light cells could be broadly considered in the rat medulla as being either in the process of active secretion or alternatively in the process of replenishing their stock of secretory material; or both these activities may operate simultaneously. The role of the mitochondria in the medullary cell must remain a hypothetical question. The fact that in any one cell the mitochondrial membranous internum is of reasonably uniform appearance in spite of considerable variation in this throughout the tissue at large, indicates that the mitochondria are closely involved in the secretory (and probably cyclical) cell changes.

Because of the difficulty of absolute identification of venous as opposed to arterial capillaries in electron micrographs, it has not been possible to evaluate, in the rat medulla, Bennett's (1941) claim that, in the cat, there is a definite cell polarity in respect of cytological components (Golgi apparatus, nucleus, granules). However as before stated, it has been a definite impression that nerve termination is found towards the capillary rather than the venous end of the cell.

SUMMARY

An electron microscopic study has been made of the rat adrenal medulla in normal and cold-stressed animals and after denervation of the gland.

Medullary cells are broadly of two sorts: a dark cell in which abundant osmiophile adrenaline granules are contained in a relatively opaque background cytoplasm: and a light cell containing fewer secretory granules. Following cold stress there is an increased number of cells partly depleted of granules.

There is a marked subendothelial space, often containing semi-opaque

material, separating parenchymal and (blood sinusoid) endothelial plasma membranes; it has inter-parenchymal cell extensions.

The mitochondria are normally compact in dark cells and often vacuolated in light cells; but in the stressed medulla light cells often contain mitochondria with a compact laminar internum.

Great variation in appearance of adrenaline granules has suggested their inception within saccular investments by an aggregation of micro-granules. In the denervated medullary cell these aggregate forms are rare and a polymorphism of granules, many of which have halos, of less osmiophile material, may suggest granule dissolution.

The medullary Golgi apparatus though frequently seen as a compact paranuclear complex, may be more diffuse.

The nature of some cytoplasmic sacs is equivocal though many can be positively identified as ergastoplasmic, Golgiform or mitochondrial in origin.

The appearance of nerve fibres and synapses is described before and after denervation.

Acknowledgments

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NOTES AND COMMENTS

CONCERNING THE ROLE OF THE SALIVARY GLAND IN THE METABOLISM OF INTRAVENOUSLY INJECTED DIIODOTYROSINE[1]

In an earlier publication from this laboratory (Tong, Taurog, and Chaikoff, 1954) it was demonstrated that intravenously injected diiodotyrosine (Ty) is rapidly removed from the circulation and deiodinated. Since rat liver and kidney tissue were quite active in deiodinating Ty *in vitro*, it was suggested that these organs were the ones primarily concerned with this function in the intact animal. Recently Fawcett and Kirkwood (1954) reported that the rapid deiodination of intravenously administered Ty does not occur in rats from which salivary glands had been removed, and from this finding they concluded that salivary glands have a major role in the extrathyroidal metabolism of organic iodine and function as "reverse thyroids." Further experiments bearing on the role of the salivary glands in the metabolism of Ty are presented here.

METHODS

Female, Long-Evans rats weighing 170 to 200 gm. were used. The removal of salivary glands was performed as follows: The animals were anesthetized with either nembutal or ether. A 3-cm. midline incision was made in the ventral surface of the neck, and all salivary and lymphatic tissues were excised with the aid of electrocautery. Special care was taken to remove all visible submaxillary, sublingual and parotid salivary tissues. The incision was closed with sutures, and the animals were allowed at least 4 hours to recover from the effects of the operation.

I^{131}-labeled Ty of about 95% radiochemical purity was prepared as described previously (Tong *et al.*, 1954). Approximately 45 or 90 μg. of this material, dissolved in 0.1 ml. of bicarbonate buffer, were injected into the tail veins of the animals while they were under ether anesthesia. After a period of 2 minutes—to allow for the initial mixing to occur—a zero-time sample of blood was taken from the tail. Subsequent samples were then taken 10, 30 and 60 minutes later.

Twenty-μl. portions of plasma from each blood sample were delivered onto strips of Whatman No. 1 filter paper and chromatographed in collidine-water-NH_3 as described elsewhere (Taurog, Tong, and Chaikoff, 1950). The main radioactive components in such chromatograms are Ty, inorganic iodide, and 3,5-diiodo,4-hydroxy-phenyllactic acid. The areas containing these components were cut out of each chromatogram, placed in vials, and assayed for I^{131} in a well-type scintillation detector.

RESULTS

In Table 1 the radioactive Ty concentrations in plasma are expressed as percentages of the values obtained for the zero-time samples. No significant difference is apparent in the disappearance rates of Ty from plasma of normal and salivariectomized rats.

In order to obtain a measure of the extent of deiodination of the injected Ty, Fawcett and Kirkwood (1954) made use in effect of the ratio,

$$\frac{\text{Plasma Ty } I^{131}}{\text{Plasma Total } I^{131}}$$

and for each time interval after the injection of the labeled Ty, they expressed the ratio as a percentage of its zero-time value. In their experiments the values for this ratio remained constant up to 60 minutes after the injection of the labeled Ty into salivariecto-

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TABLE 1. DISAPPEARANCE OF INTRAVENOUSLY INJECTED I^{131}-LABELED DIIODOTYROSINE FROM PLASMA OF INTACT AND SALIVARIECTOMIZED RAT

Animal no.	Plasma diiodotyrosine as per cent of initial value			
	Zero time	10 minutes	30 minutes	60 minutes
Intact				
1	100	25.8	9.2	3.9
2	100	33.4	13.5	6.6
3	100	33.0	12.1	4.2
4	100	37.8	16.4	7.4
5	100	41.0	15.9	5.4
6	100	33.3	12.3	4.9
Mean ±S.D.*		34.1±5.4	13.2±2.4	5.4±1.6
Salivariectomized				
1	100	28.2	11.9	5.7
2	100	27.3	10.6	4.6
3	100	32.8	11.7	4.9
4	100	23.9	8.9	4.3
5	100	37.4	15.6	6.6
6	100	32.3	13.9	4.7
7	100	36.1	13.2	4.7
8	100	39.9	12.4	4.9
Mean ±S.D.*		32.2±5.1	12.3±1.9	5.1±2.0

* S.D. $= \sqrt{\frac{\Sigma d^2}{n}}$.

mized rats, whereas in normal rats the values for the ratio decreased to less than 40% of the zero-time value. Such calculations were also made for our data, and the results are recorded in Table 2. No difference was noted in the results obtained for normal and salivariectomized rats.

TABLE 2. PLASMA DIIODOTYROSINE I^{131} AS PER CENT OF PLASMA TOTAL I^{131} IN INTACT AND SALIVARIECTOMIZED RATS

Animal no.	$\frac{\text{Plasma diiodotyrosine } I^{131}}{\text{Plasma total } I^{131}}$ as per cent of initial value			
	Zero time	10 minutes	30 minutes	60 minutes
Intact				
1	100	47.5	21.1	11.1
2	100	57.3	25.9	20.9
3	100	44.2	20.9	10.8
4	100	53.1	27.6	15.7
5	100	51.4	28.7	12.5
6	100	46.0	21.7	11.3
Mean ±S.D.*		49.9±4.4	24.3±3.0	13.7±3.6
Salivariectomized				
1	100	53.9	26.3	14.1
2	100	52.5	33.1	13.3
3	100	51.0	25.1	11.4
4	100	47.0	21.8	12.8
5	100	56.1	29.2	15.2
6	100	48.4	26.2	11.9
7	100	55.8	26.0	11.1
8	100	57.8	24.5	12.1
Mean ±S.D.*		52.8±3.6	26.5±3.1	12.7±1.3

* S.D. $= \sqrt{\frac{\Sigma d^2}{n}}$.

SUMMARY

Normal and salivariectomized rats were compared with respect to the rate at which diiodotyrosine was removed from plasma and deiodinated. The results presented here fail to support the view that the salivary glands play a major role in the metabolism of diiodotyrosine.

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AWARDS OF THE ENDOCRINE SOCIETY

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. In 1955 the Medal was presented to Dr. Carl R. Moore.

The Ciba Award

The Ciba Award to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945, Dr. Jane A. Russell; 1946, Dr. Martin M. Hoffman; 1947, Dr. Choh Hao Li; 1948, Dr. Carl Heller; 1949, Dr. George Sayers; 1950, Dr. Oscar M. Hechter; 1951, Dr. Albert Segaloff; 1952, Dr. Seymour Lieberman; 1953, Dr. Sidney Roberts and Dr. Claire Szego (Mrs. Roberts); 1954, Dr. Isodore M. Rosenberg; 1955, Dr. Jack Gross. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-fourth months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

The Ayerst, McKenna and Harrison Fellowship

This fellowship was established in 1947. It is designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. The fellowship is awarded on alternate years (1955, 1957, etc.) and the stipend which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree or candidates for either of these degrees are eligible for appointment.

Applicants must submit the following information:

(1) Evidence of scientific ability as attested by studies completed or in progress.

(2) Recommendations from individuals familiar with the candidate and his work.
(3) A proposed program of study.
(4) Acceptance of the individual by the head of the department in which the Fellowship will be held.
(5) A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society established a category of Scholars. These grants have been made available through the generosity of The Schering Corporation and The Upjohn Company, and will be awarded to established investigators and teachers in the fields of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual, and shall be granted on the basis of proposals submitted by the applicant and such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of The Endocrine Society; the Ciba Award; and the Ayerst, McKenna and Harrison Fellowship may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 N. Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than November 1st each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by November 1, 1955.

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1955.